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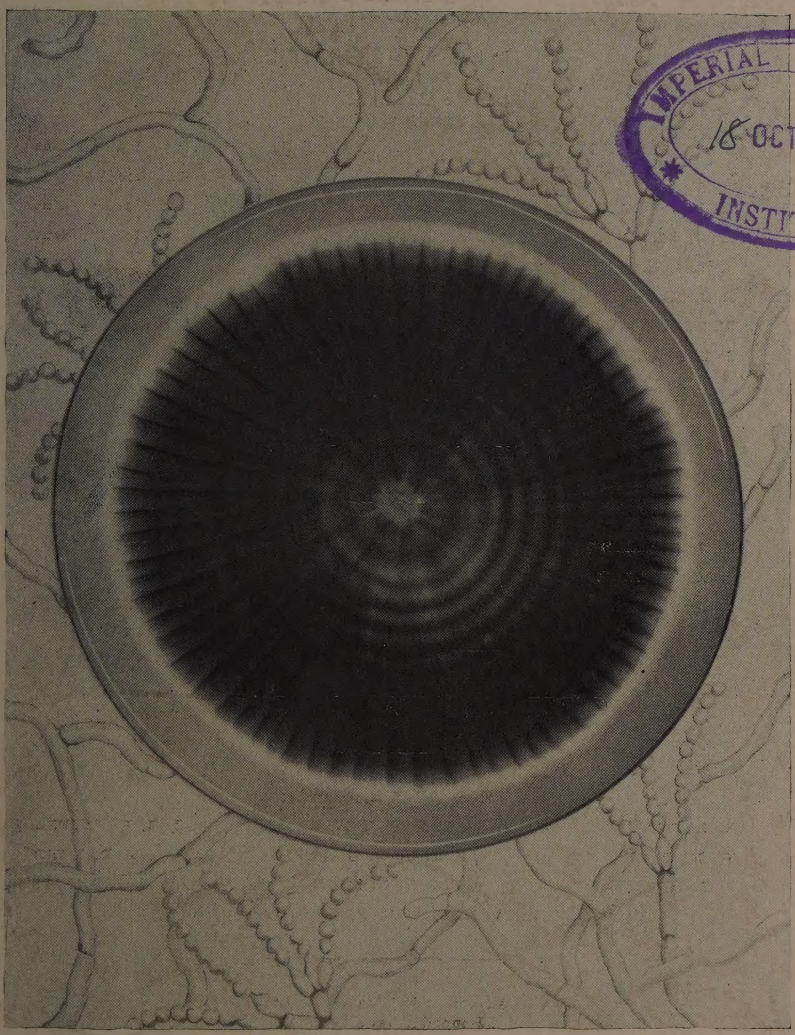
THE HAWAIIAN PLANTERS' RECORD



Penicillin, one of the most remarkable therapeutical agents ever discovered, is produced by this simple mold. A colony and the microscopic appearance of the mycelium and spores of the mold are illustrated. The culture of this mold and the preparation of active penicillin solutions and surgical dressings and their topical use in Hawaii are reported in this issue.

FIRST QUARTER 1945

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Studies With *Penicillium Notatum* Westling in Hawaii

By C. W. Carpenter, D. M. Weller, and J. P. Martin

The discovery of penicillin by Dr. Alexander Fleming followed by clinical reports of its unique therapeutic properties stimulated world-wide research in "biotherapy." Discussions of the production, purification, potency and clinical aspects of penicillin have occupied prominent places in the popular journals, the press, and technical periodicals. The more significant contributions in the literature are reviewed. Authentic penicillin-producing strains of Penicillium notatum *were obtained from mainland laboratories and grown on different media in various containers to obtain maximum yields of penicillin. Inoculated surgical dressings and active penicillin culture solutions were prepared for topical therapy and tested for potency, preferably against the specific pathogen concerned, prior to distribution to physicians. One hospital on Hawaii, Maui, and Kauai prepares such active material, in cooperation with the medical adviser of the Association and with this Station, for the convenience of plantation and other physicians. Many physicians and technicians of the armed forces have been given cultures of* P. notatum *and instructions for preparing active penicillin solutions and inoculated dressings as a result of which penicillin preparations are being made at sea as well as ashore. One medical unit of the U. S. Navy which has been preparing such materials for the past eight months has now been officially designated for expansion of the project to supply the advanced military areas as well as the local associated medical units. Materials prepared by, or in cooperation with this Station, have satisfied the urgent demand for topical therapy while the use of purified penicillin remained stringently controlled by the War Production Board. References to clinical cases treated topically with penicillin solutions and dressings in Hawaii are included.*

Introduction

The germ conception of the cause of disease was established on a firm scientific basis by Pasteur in the decade 1860–1870 and since that time scientists have eagerly

sought a substance which could be used without harmful effects in the human system to destroy pathogenic bacteria. Partial success in this hopeful quest was realized with the discovery in 1932 by Domagk (5, 6) of the therapeutic value of sulfonamid followed by the amazing success of this compound and its derivatives in the period 1932–1940. These compounds while not completely free from toxicity in the human system are nevertheless used successfully in treating certain infectious bacterial diseases; with other bacterial infections they are ineffective. Chemotherapy of protozoan infections is of ancient origin but the practical application of chemotherapy in systemic bacterial diseases had its first outstanding success in the period mentioned. The performance of the sulfa compounds renewed interest in the science of bacterial chemotherapy and shaped the course of research in this field.

The discoveries of the sulfa compounds and penicillin were neither spontaneous nor accidental. The discovery of the antibacterial properties of sulfonamid was preceded by studies of the physiology and growth requirements of bacteria. The basic compound para-amino-benzene sulphonamid, first prepared by a student, P. Gelmo (13) in Vienna in 1908, found little practical use until Domagk published his observations on its therapeutic value. The discovery of penicillin, a product of the mold *Penicillium notatum* Westling, by Fleming (8) in 1929, and its application in his researches, were brilliant triumphs of inductive reasoning. The isolation and practical development of penicillin were equally triumphs of cooperative endeavor in the field of applied science. Fleming's discovery has been called accidental. It was in reality the routine follow-up of a chance observation closely related to his search for substances unfavorable to bacterial growth. The orientation and integration of earlier successes and failures provided the foundation for these extraordinary attainments in bacterial chemotherapy.

The principle of association among microorganisms was discussed by Pasteur (16) in 1863 with particular reference to the coexistence of aerobic and anaerobic bacteria. DeBary in 1879 observed and commented upon the antagonisms resulting from the competition of various bacteria for the same nutrients. Such antagonistic behavior among organisms was termed antibiosis by Ward (18). Prior to the discovery of penicillin, antibiosis—offense as a means of defense among organisms—was a fascinating subject of inquiry. It was a matter of great interest but had found no practical application in disease control. Stimulated by the noteworthy performance of penicillin the search for similar antibiotic substances—a search not restricted to the lower forms of life—became world-wide. A number of such substances produced by molds and bacteria have been discovered, some of which may prove of practical value. Penicillin is widely applicable yet relatively non-toxic. It is unique.

Penicillium notatum is not a common species but is one of several hundred representatives of the genus Penicillium, an ubiquitous group of fungi with inconspicuous white mycelia when young but which become green to powdery blue with the development of multitudes of microscopic spores when mature. By no means are all green molds Penicillia. The Penicillia are, however, among the most common green molds associated with the spoilage of fruit, meat, bread and other cereal products. Two species are economically important, being essential in the ripening of Roquefort and Camembert cheeses—the delectable piquant flavors of which are due to volatile fats hydrolyzed by the enzymes of the specific mold used. The common idea that any green mold, at random, occurring on bread, fruit, leather, etc., produces penicillin is erroneous.

Many of the numerous species of Penicillium may be identified only by considering their physiological reactions in addition to the morphological characteristics which serve to place them in a particular group. At maturity the spores of Penicillia develop in great abundance on the brush-like spore organs and when dry these dust-like spores upon the slightest disturbance drift away freely in air currents. They are omnipresent and promptly germinate to form mycelia in the presence of moisture and a suitable medium which may consist of a remarkable diversity of materials, organic or inorganic. Such molds as the Penicillia are the notorious weeds of the kitchen and of industrial plants as well as of the laboratory; in the latter enforcing a meticulous technique wherever pure cultures of bacteria and fungi are being maintained.

Discovery, Properties, and Production of Penicillin

Discovery of Penicillin:

Dr. Alexander Fleming (8), at St. Mary's Hospital, London, in 1929, while working with *Staphylococcus aureus,* a common pus-forming microbe, observed a contamination in the form of a mold colony on one of his culture plates. Such an occurrence is not uncommon in the laboratory especially when plate cultures are retained for study over an extended period. Fleming's attention was attracted by a clear zone or halo surrounding the fungus invader wherein colonies of *S. aureus* had not developed, whereas elsewhere on the plate the Staphylococci had developed normally. Pursuing his investigation of this phenomenon of antagonistic activity Fleming concluded that the fungus which he recognized as a species of Penicillium produced some substance which inhibited the growth of *S. aureus.* He applied this principle in the isolation of *B. influenzae,* eliminating *S. aureus* from his cultures by growing the desired organism in a medium containing a crude solution of the inhibitory substance to which *B. influenzae* was not sensitive. The inhibitory substance was named penicillin by Fleming. The mold was subsequently identified as *Penicillium notatum* Westling.

Fleming used broth in which *P. notatum* had been grown to treat a few patients topically for infections and reported that the results appeared to be superior to the usual treatment with standard antiseptics. He stated that the toxicity of penicillin to animals was very low; constant irrigation of large infected areas on man were followed by no adverse symptoms, and irrigation of the human eye every hour for one day proved non-irritating. *In vitro* the crude solution caused no greater interference with leucocytic function than ordinary broth. Among the properties of the antibacterial substance produced by *P. notatum,* as determined by Fleming, which served as a basis for subsequent investigations are the following: it is resistant to heating at 56° C. and at 80° C. for one hour; boiling for a few minutes has no effect, while boiling for one hour reduces the activity by more than 75 per cent if the fluid is alkaline, with less reduction in potency if the fluid is neutral or slightly acid. Penicillin is soluble in water and weak saline solutions. Its potency may be retained for four days at room temperature after which it rapidly diminishes, but the material is more stable if the alkaline broth is adjusted to pH 6.8. Fleming tabulated the inhibitory activity of penicillin on various bacteria *in vitro,* and listed the following bacteria as non-sensitive: members of the coli-typhoid group, intestinal bacteria (*B. pyocyaneus, B. proteus* and *V. cholerae*), enterococcus, some

gram-negative cocci, Friedlander's bacillus, and *B. influenzae*. Sensitive bacteria listed included: *pneumococci, gonococci, meningococci* and *B. diphtheriae* (1–10 dilution of the unconcentrated filtrates).

Clutterbuck, Lovell and Raistrick (3) studied the strain of *Penicillium* used by Fleming and in 1932 reported it to be closely related to *P. notatum* Westling. They confirmed Fleming's observations on the resistance or susceptibility of various bacteria to penicillin. They found that filtrates of the crude culture solutions had retained their potency after 16 weeks at 9° C., but that contaminated filtrates lost potency immediately. These investigators demonstrated that penicillin in acid solutions is soluble in ether.

Fleming (9) compared the potency of an impure preparation of penicillin (30 per cent active principle) with sulfathiazole and sulfapyridine against *Streptococcus pyogenes* and *Staphylococcus aureus* and found it, weight for weight, four times as potent as sulfathiazole and twenty times as potent as sulfapyridine.

In 1938, Professor Howard Florey (10) and his colleagues, at the Sir William Dunn School of Pathology, Oxford University, began to investigate antibacterial substances produced by fungi and bacteria. While it was known that organisms may arrest, or inhibit, the growth of others in cultures, this principle had not been definitely applied *in vivo*. As this research progressed collaborators in chemistry, bacteriology, and medicine were called in to assist in the extraction and purification of penicillin and in testing penicillin against pathogenic organisms first in animals and later in man. It was soon established by Florey and Florey (11) that, because of its remarkably low toxicity and its amazing properties, penicillin offered great possibilities in bacterial chemotherapy. Up to this time only small quantities of penicillin had been produced. It was essential to increase production before penicillin could be used extensively.

Due to war conditions in England, and the urgent need for larger quantities of penicillin, the British workers asked for aid from the United States Government and from various American scientific institutions. Appreciating the potential possibilities of penicillin and the necessity for increasing production the various organizations immediately responded. The essential research work was soon under way. Large plants costing a total of many millions of dollars have been constructed in the United States and Canada for the production of penicillin under Government control.

Properties of Penicillin:

Penicillin, a complex organic acid, is obtained mostly at present from the molds *Penicillium notatum* and *P. chrysogenum*. Studies on the chemistry of penicillin are being conducted in many laboratories, as a result of which it is hoped that the chemists will establish the structural formula and, in the near future, be able to prepare it synthetically. In pure form penicillin is colorless. The sodium, potassium, and ammonium salts of penicillin are hygroscopic and must be stored under dry conditions. All forms of penicillin must be held at low temperatures. Penicillin is labile in both acid and alkaline solutions and most stable at pH 6.5.

According to prevailing opinion penicillin prevents the reproduction of bacteria by fission, thus controlling the increase in numbers of the organisms, an effect known as bacteriostasis. However, according to some workers penicillin may cause lysis (cell destruction); action of this type is called bactericidal. According to

Florey and Jennings (10) penicillin at dilutions of 1:20–30,000,000 inhibited the growth of *Staphylococcus aureus,* while a partial inhibition was reported at a dilution of 1:160,000,000. At dilutions of 1:4–8,000,000 penicillin in its purest form completely inhibited the growth of the following organisms: *S. aureus, Streptococcus pyogenes* (group of pus-forming organisms), *Streptococcus viridans,* and *Clostridium welchii* (commonly found in cases of gas gangrene).

Some organisms are able to develop in the presence of penicillin and these are known as penicillin-fast organisms. Penicillin has no effect on *B. pyocyaneus* nor in general on gram-negative bacilli, some of which frequently infect wounds.

Peniciliin has little or no effect on either red blood corpuscles (erythrocytes) or white blood corpuscles (leucocytes), thus allowing the latter to function as normal defense mechanisms. Penicillin leaves the body quickly in the urine and may be recovered from it; penicillin so recovered has a high antibacterial titer.

Production of Penicillin:

In the laboratory the mold *P. notatum* is grown on various liquid media in containers which may vary greatly in size and shape. Erlenmeyer flasks, prescription bottles, gallon jugs, and even large covered pans are some of the kinds of containers used for culturing the mold. It has been shown that a higher concentration of penicillin in the culture solution is obtained when the liquid on which the mold is cultured is not more than 2 cm. in depth. This technique of growing the mold known as the "surface-culture method" has certain advantages over other methods, the outstanding ones being: higher yields of penicillin; simple equipment; and smaller losses from contamination.

The yield of penicillin from liquid cultures is very low, approximately one ounce of penicillin being obtained from 125 gallons of solution. In the commercial production of penicillin, therefore, very large volumes of culture solution must be handled and the greatest drawback with the tedious surface-culture method is the large number of containers and the incubation space required to take care of 1000 gallons or more of solution. However, this method, due to its advantages over other methods, is still in use.

Other methods for growing the mold are being studied and several offer considerable promise. In the bran method the mold is grown on layers of moist sterilized bran for several days and the penicillin is then extracted with a suitable organic solvent.

A number of commercial plants are now using the "submerged-culture method" for growing strains of *P. notatum* adapted to this type of fermentation. Instead of using many small containers large tanks or vats are used, the latter having a capacity up to 1000 gallons or more. As the name of the method implies the fungus is grown in an aerated solution rather than on the surface. The strains of the mold used in the surface-culture method were found to be less efficient in the submerged-culture method. New strains adapted to this method had to be found. During the growth of the mold the solution must be agitated and aerated with sterile air. Contamination throughout the entire system has to be prevented so that the mold may be grown under aseptic conditions. The chemical control in any industrial plant producing penicillin must be exact. The preparation of the culture solution, and maintaining the proper hydrogen-ion concentration of the solution for maximum mold growth and penicillin production are only a few of the important phases of

the work. Commercial production by the tank method was really a remarkable achievement.

In both the surface- and submerged-culture methods the mold is grown for from 5 to 10 days, during which time the penicillin secreted by the fungus accumulates in the solution. When the concentration of penicillin is highest, as determined by the Oxford ring test (discussed later), the extraction process begins.

Considerable information relative to the extraction and purification of penicillin from the unconcentrated filtrates of *P. notatum* has been published (1), but the methods are constantly being improved; the production process being still under governmental jurisdiction, the details are not available. The production and allocation of penicillin were placed under the direct control of the War Production Board on July 16, 1943.

The final product for clinical use is the sodium salt of penicillin, a hygroscopic and labile substance with which extreme precautions must be taken in drying, packaging, and storing. The calcium salt which is more stable is now rapidly coming into use.

A bacteriological technique of the highest standard is required in the production of penicillin. Pure culture methods must be maintained at all times. Contaminations anywhere along the line may result in no yield of penicillin or a product of

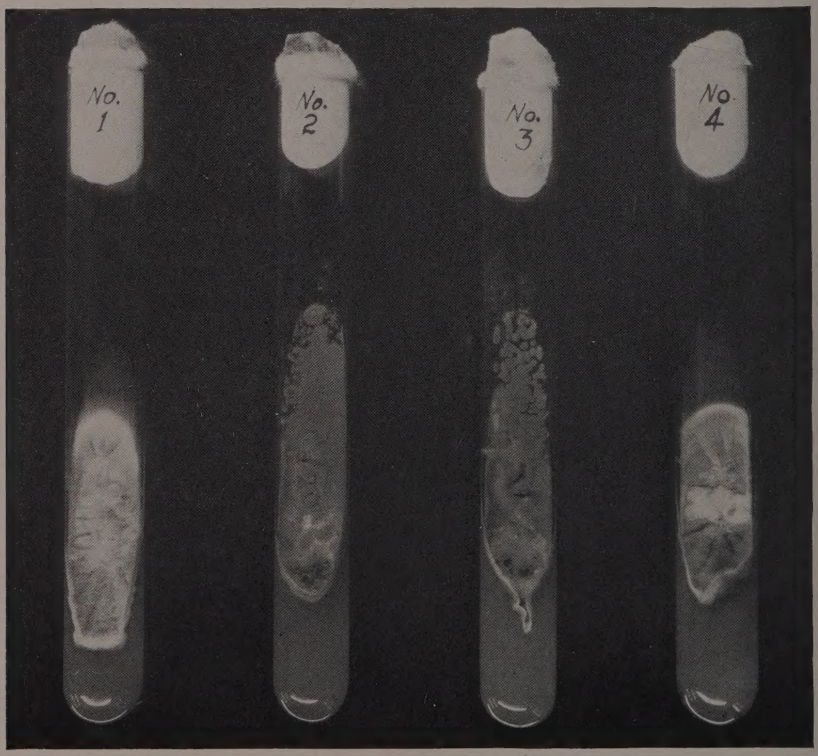

Fig. 1. *P. notatum,* strains 1, 2, 3, and 4, grown on nutrient agar slants, Medium No. 1, age 8 days.

low quality. A number of organisms produce enzymes which have the power to destroy penicillin and the necessity for excluding such organisms is obvious.

Studies in Hawaii

During the early part of July 1943 Dr. H. L. Lyon requested that, if possible, different strains of *Penicillium notatum* which have been used in the production of penicillin be obtained so that their growth under local environmental conditions might be studied. Local strains of *P. notatum,* which might produce high yields of penicillin, were also sought.

Four strains of *P. notatum* were obtained from two mainland laboratories* (Figs. 1, 2, and 3). Each strain had been used in the production of penicillin (Figs. 4 and 5). For convenience the strains were numbered 1, 2 (No. 824), 3

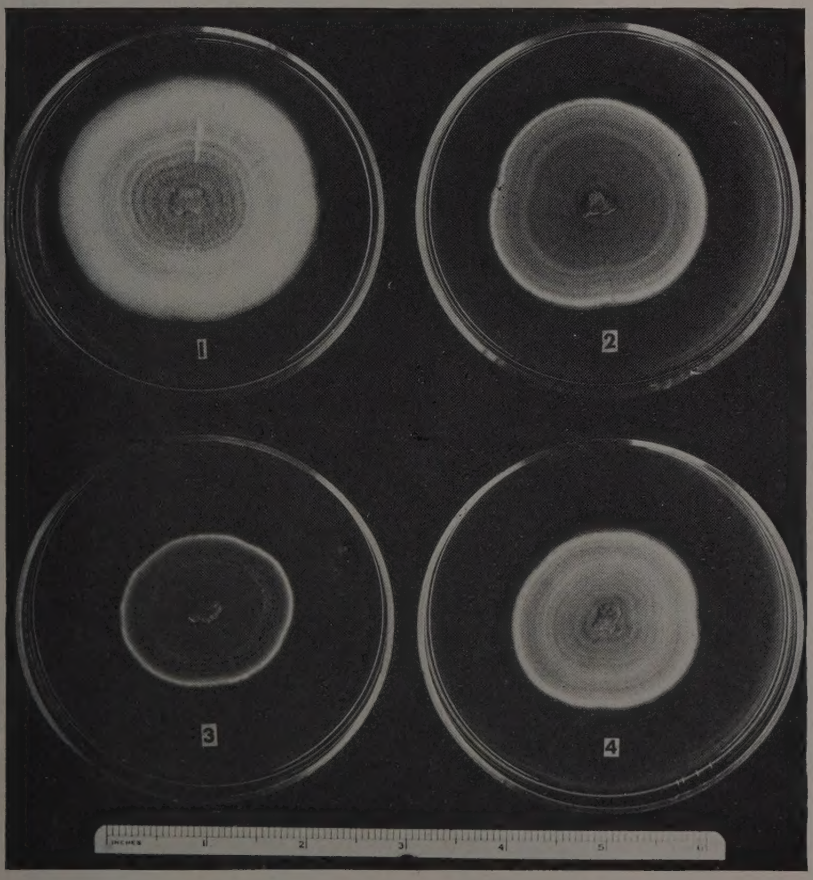

Fig. 2. Individual colonies of strains 1, 2, 3, and 4 of *P. notatum* grown on agar plates, Medium No. 1, age 11 days.

* Three strains (Nos. 824, 830, and 1249–B21) were obtained from Dr. K. B. Raper, Northern Regional Research Laboratories, Peoria, Ill., and one strain from Dr. Walter Kocholaty, University of Pennsylvania, Philadelphia, Penn.

(No. 830), and 4 (No. 1249–B21). Strain No. 2 is a subculture of the original Fleming strain while strain No. 4 had been used extensively in the surface-culture method for penicillin production. It was soon learned, by the Oxford ring test (Figs. 7 and 8), that strain No. 4 yielded the greatest concentration of penicillin in surface cultures and, therefore, further studies were conducted chiefly with this strain. Stock cultures of the four strains have been maintained. Cultures of strain No. 4 and instruction in culture methods have been given to physicians, technicians and medical service personnel.

The optimum temperature for growing *P. notatum* is about 72° F. which corresponds closely to our average room temperatures. It was soon demonstrated that our prevailing temperature was very satisfactory for growing the fungus without incubators.

Several local cultures of *Penicillium* sp., resembling *P. notatum,* were isolated from various sources such as bread, banana, citrus fruit (orange), etc. One of these cultures exhibited antibacterial properties when grown both in surface and submerged culture and tested by the ring test, as well as by growing the fungus on poured plates of *S. aureus.* However, it was not found to be superior to strain No. 4.

Studies with *P. notatum* were limited chiefly to growing it on various liquid

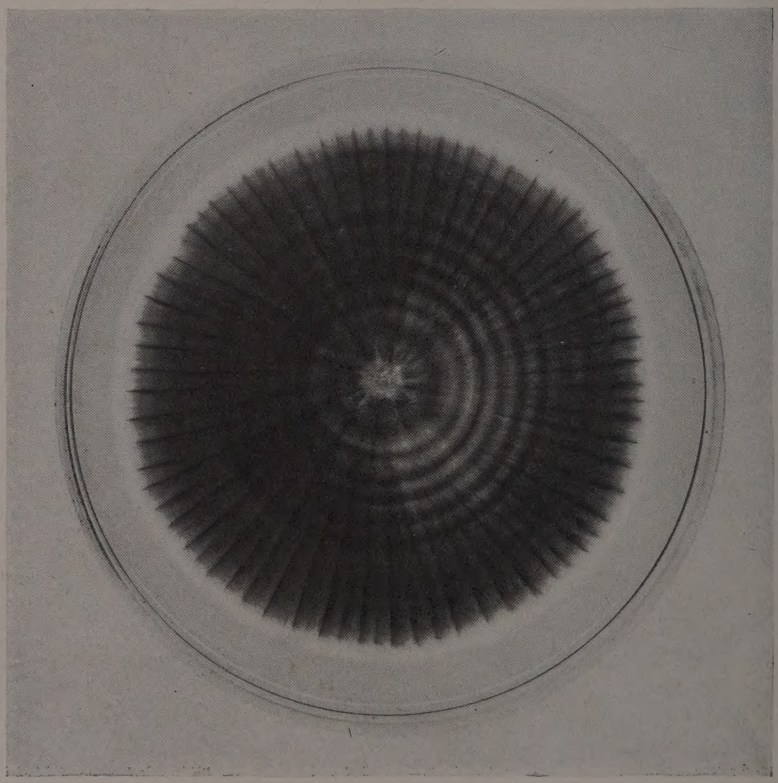

Fig. 3. A large colony of *P. notatum*, strain No. 4, age 13 days, grown on Czapek-Dox agar.

media which might yield higher concentration of penicillin as determined by the Oxford ring test, and preparing penicillin solutions and inoculated gauze dressings. It should be recorded in explanation of the comparatively low titer of our culture solutions, that corn steeping liquor which Moyer discovered to be a valuable supplement to the Czapek-Dox medium which increased the penicillin yield at least tenfold, according to Coghill (4), was not available in Hawaii, nor have we found a comparable substitute. Consequently we have followed the general culture methods of Abraham *et al* (1) and have observed comparable yields of penicillin in our culture solutions.

We were very fortunate in having the interest, advice, and cooperation of Dr. Nils P. Larsen, Medical Adviser, H.S.P.A., who made the information from these investigations available to plantation physicians. Dr. Larsen made arrangements whereby one hospital on each island maintained stock cultures of *P. notatum* and prepared penicillin solutions and gauze dressings. The plantation physicians on each island were then able to obtain information and materials without delay. As the work developed requests were received from local civilian and service physicians

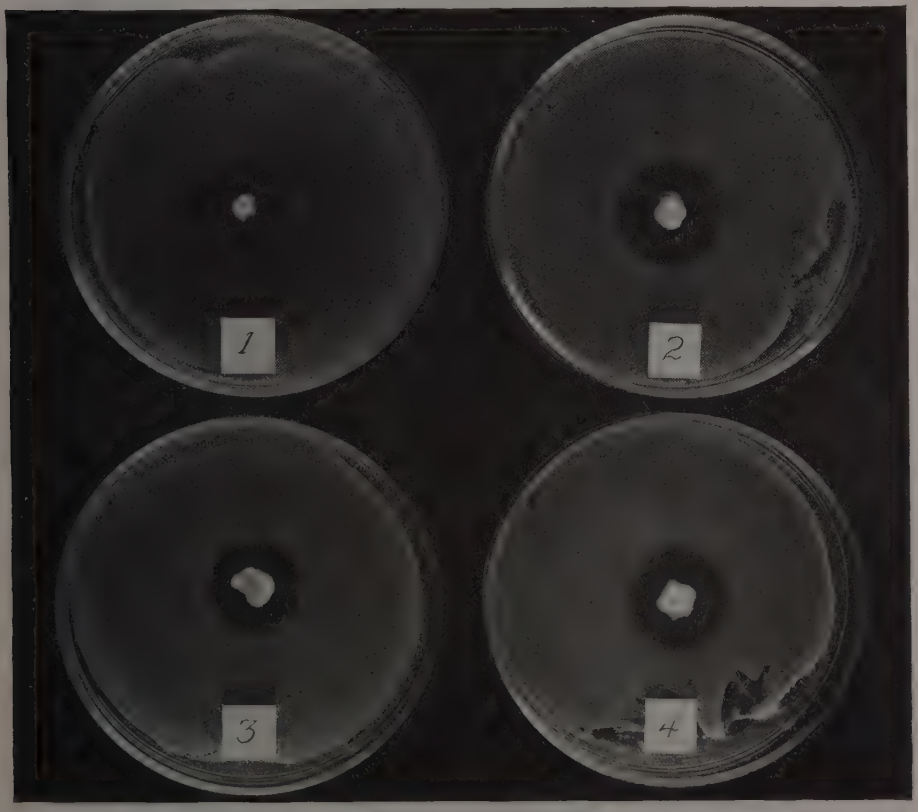

Fig. 4. Inhibition zones on plates of *S. aureus* (Medium No. 2) produced by 24-hour-old cultures of *P. notatum*, strains 1, 2, 3, and 4. At the end of 24 hours the plates were flooded with a water suspension of *S. aureus*, and incubated overnight.

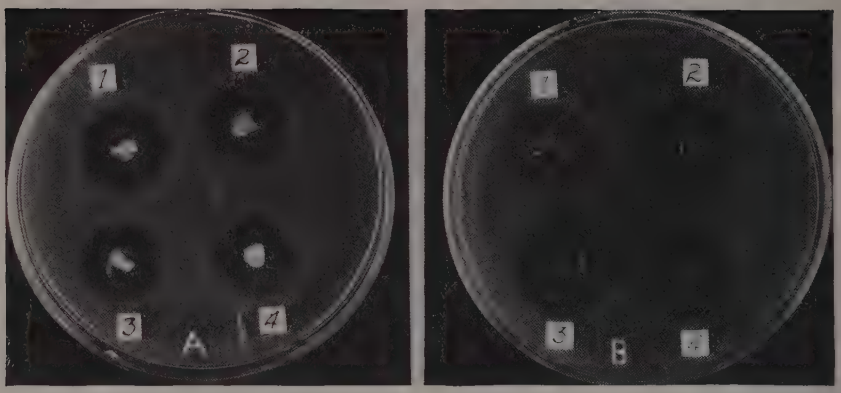

Fig. 5. *A*—Inhibition zones on poured plates (Medium No. 2) of *S. aureus* produced by transplanted portions of 1-day-old cultures of *P. notatum,* strains 1, 2, 3, and 4. *B*—Inhibition zones on plates of *S. aureus* formed by agar discs taken from the edges of 6-day-old colonies of strains 1, 2, 3, and 4, respectively.

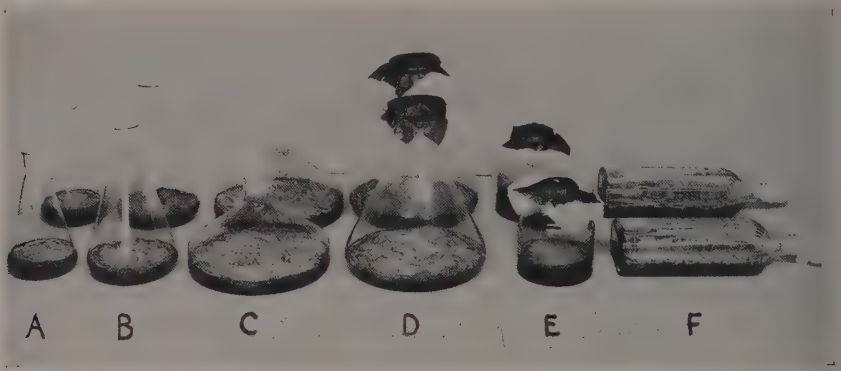

Fig. 6. Containers used for surface culture of *P. notatum.* *A*—500-ml. flask; *B*—1000-ml. flask; *C*—1860-ml. flask (low form); *D*—2800-ml. flask (wide mouth); *E*—500-ml. Baxter bottle; and *F*—32-ounce prescription bottle.

for penicillin solutions and dressings for clinical uses, the preparation and distribution of which were supervised by Dr. Larsen. Later, when limited quantities of pure penicillin became available for civilian use local requests for penicillin solutions and gauze dressings were reduced.

Cultural Studies:

Solid Media: The four strains of *P. notatum,* upon their receipt from the mainland, were immediately transferred to standard nutrient agar prepared as follows:

Medium No. 1	Peptone	5 grams
	Beef extract	3 "
	Sucrose	20 "
	Agar	18 "
	Distilled water to make	1000 cc.
	pH adjusted to 6.5	

Each strain made an excellent growth and sporulated freely on slants, Fig 1, and on plates, Fig. 2, of this medium, which was also found to be highly satisfactory for maintaining stock cultures and for conducting other studies where a solid medium was required. Czapek-Dox solution (Medium 3) plus 1.5 per cent agar was also used for stock cultures. These two media were superior to Medium No. 2 for maintaining the vitality of the fungus.

The agar medium used for stock cultures and test plates of *S. aureus* was prepared according to the following formula:

Medium No. 2	Bacto nutrient agar dehyd. (Difco)	23 grams
	Neopeptone (Difco)	5 "
	Dextrose	10 "
	Distilled water to make.....	1000 ml.
	pH adjusted to 6.8	

Surface Culture Method—Liquid Media: The first synthetic medium used for culturing *P. notatum* in these studies was the modified Czapek-Dox solution, used by Clutterbuck *et al* (3) in 1932 and by Abraham *et al* (1) in 1941. This medium was prepared as follows:

Medium No. 3	$NaNO_3$	3.0 grams
	KH_2PO_4	1.0 "
	KCl	0.5 "
	$MgSO_4 \cdot 7H_2O$	0.5 "
	$FeSO_4 \cdot 7H_2O$	0.01 "
	Glucose (sirup)	40.0 "
	Distilled water to make ...	1000 ml.
	pH adjusted to 6.5	

Each of the four strains of *P. notatum* made excellent growth on this medium and gave good yields of penicillin as measured by the ring test. Subsequently this medium (as well as others) was made from stock solutions in which the above salts were contained in a hundred times the required concentrations; 10 ml. were pipetted from each stock solution for each liter prepared. In this way various media were more quickly and accurately prepared. The oxidation of the stock solution of ferrous sulfate to the ferric form was delayed by adding 1.015 grams of Rochelle salts ($KNaC_4H_4O_6 \cdot 4H_2O$) to each liter of the stock solution.*

The different types of containers shown in Fig. 6 were employed in testing the various culture media for the maximum yield of penicillin from *P. notatum* by the surface-culture method. These containers and the amount of solution required in each to provide a maximum depth of 2 cm. are listed in the following table:

Type of container	Ml. per container
A— 500 ml. Erlenmeyer flask.....	150
B—1000 ml. Erlenmeyer flask.....	200
C—1860 ml. flask, low form.....	500
D—2800 ml. Erlenmeyer flask, wide mouth...	500
E— 500 ml. Baxter bottle	200
F— 32 ounce (946 ml.) prescription bottle..	250

* The addition of Rochelle salts was made at the suggestion of Paul Gow of the Chemistry department.

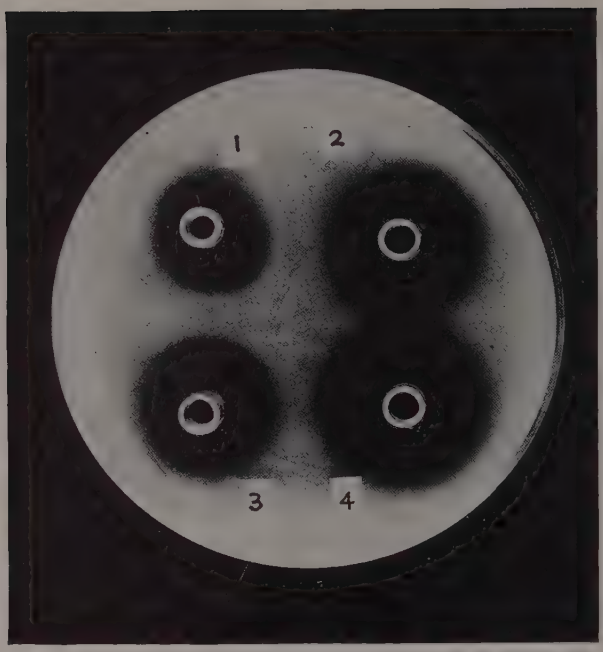

Fig. 7. Showing zones of inhibition of *S. aureus* on poured plates (Medium No. 2) formed by 10-day-old culture solutions (Medium No. 4), surface method, of *P. notatum*, strains 1, 2, 3, and 4. Strain No. 4 gave the greatest yield of penicillin as measured by the Oxford ring test.

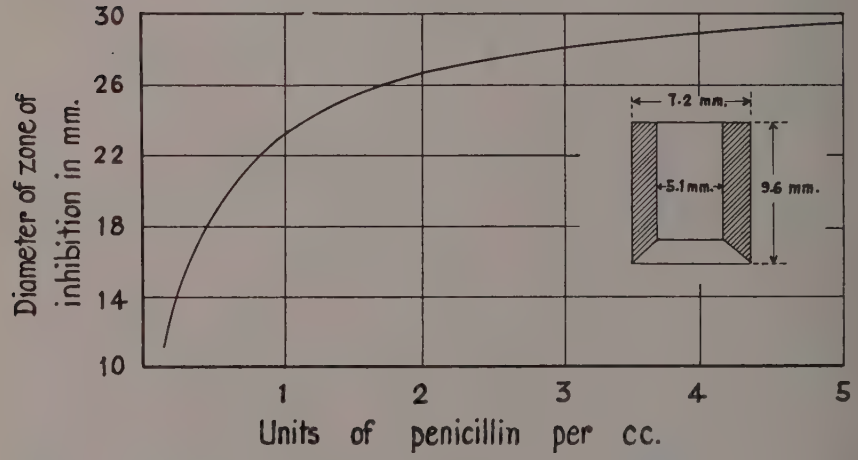

Fig. 8. Graph used for measuring the concentration of penicillin in solutions. The dimensions of the cylinders used in the Oxford cylinder method are also shown (after Abraham *et al* [1]).

Fig. 9. Showing 5-day-old cultures of *P. notatum* (Strain No. 4) on Medium No. 4 inoculated with spores of different ages produced on different culture media.

A-1, *A*-2, inoculum from 1-month-old culture on Med. No. 2

B-1, *B*-2, inoculum from 1-month-old culture on Med. No. 1.

C-1, *C*-2, inoculum from 1-week-old culture on Med. No. 2.

D-1, *D*-2, inoculum from 1-week-old culture on Med. No. 1.

While little difference in growth is seen between *C*-1, *C*-2, and *D*-1, *D*-2, this difference is emphasized as the age of the cultures (from which the inoculum is taken) increases.

A corresponding difference in titer is obtained in the solutions of such cultures as is shown by the inhibition zones in the Petri dishes *A, B, C,* and *D.*

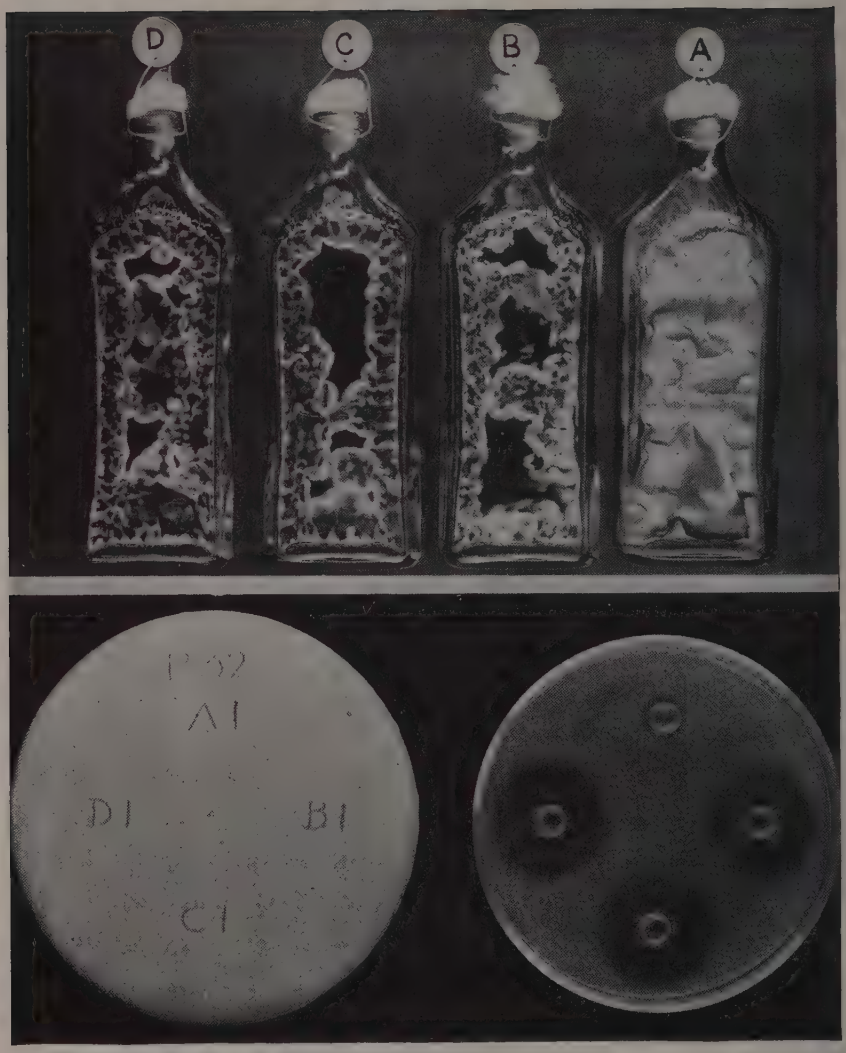

Fig. 10. Showing the effects on vegetative growth and sporulation of *P. notatum* (Strain No. 4) when grown for 6 days on Medium No. 3 with different amounts of yeast extract added, and the difference of titer of these liquids as indicated by the relative size of inhibition zones produced by them in the Oxford ring test. Yeast extract: A, 0%; B, 0.5%; C, 1.0%; and D, 2.0%.

For specific tests some containers were found to be superior to others. The 500 ml. Baxter bottle (Type E) was used with considerable success for culturing the mold and for inoculated dressings, as well as for storing the filtered penicillin solutions. The 32-ounce prescription bottle (Type F) was finally adopted as the most desirable type available for growing the mold. This bottle has a small mouth, which reduces the chances of contaminations and, being graduated, the desired quantity of liquid medium can be measured directly in the container. When the bottle is placed in a horizontal position, optimum conditions, *i.e.,* maximum surface area and mini-

mum depth of medium, are maintained for the growth of the mold and the securing of the maximum titer of solution.

A measured quantity of the liquid medium was added to the type of container used, as indicated in the table above, and sterilized at 15 pounds pressure for 20 minutes. The solution was then inoculated with a spore suspension prepared from a 6- to 10-day-old culture of *P. notatum*. A higher titer resulted when media were inoculated with spores from young cultures (6-10 days old, Fig. 9). On the second day the surface of the liquid was covered with small individual colonies which soon enlarged. On the third day the colonies now with green-colored centers began to coalesce and by the fourth or fifth day the entire surface was covered and patches of blue-green color were visible, the color being due to the development of spores. About the sixth or seventh day the mold had developed into a heavy, wrinkled, compact, dark green-blue mycelial mat; somewhat later the color became grey. Shortly after the fungus had completely covered the surface small yellowish-to-amber droplets appeared on the surface of the mold. The lower surface of the mycelial pad which was in contact with the liquid was of a yellowish-brown color as was also the liquid itself.

Satisfactory yields of penicillin from *P. notatum* as determined by the Oxford cup test with *Staphylococcus aureus* were obtained with the above mentioned Czapek-Dox modified solution, Medium No. 3. The addition of yeast extract not only improved the rate of production of penicillin as suggested by Abraham *et al* (1) but also the titer; one per cent of this material was found superior to 0.5 and 2 per cent for rate of production and maximum titer (Fig. 10).

The optimum amount of zinc sulfate per liter was found to be 0.001 gram (1 p.p.m. $ZnSO_4$) corresponding to the amount recommended by Foster, Woodruff and McDaniel (12). According to these investigators "... zinc simply accelerates the formation of penicillin, probably because it accelerates the rise in pH through oxidation of gluconic acid by the mold."

It was observed that in Medium No. 3, when supplemented with yeast extract and zinc, the titer reached a maximum in 8 to 10 days whereas without these supplements 12 to 16 days were required. The titer was increased by either yeast extract or zinc alone and there was an additive response when both were present in the medium. Medium No. 3 with the addition of one per cent yeast extract and one p.p.m. of $ZnSO_4$ was adopted as a basic medium. It is referred to as the "Basic Medium" or Medium No. 4 in further experiments and also in the production of solutions and dressings for topical application. The Basic Medium was prepared as follows:

Medium No. 4 "Basic Medium"	$NaNO_3$	3.0 grams
	KH_2PO_4	1.0 "
	KCl	0.5 "
	$MgSO_4 \cdot 7H_2O$	0.5 "
	$FeSO_4 \cdot 7H_2O$	0.01 "
	$ZnSO_4$	0.001 "
	Yeast extract	10.0 "
	Glucose (sirup)	40.0 "
	Distilled water to make	1000 ml.
	pH adjusted to 6.5	

The titer of culture solutions was found to be less when 2 and 4 per cent, respectively, of raw sugar were substituted for the 4 per cent of glucose (sirup) in the Basic Medium. Moreover when active solutions containing raw sugar in substitution for glucose were stored under refrigeration they lost titer sooner than the Basic Medium.

The medium used by Foster *et al* (12) which contained twice the amount of sodium nitrate (6 g. per liter) and 15 times the amount of ferrous sulfate (0.15 g. per liter), and one p.p.m. $ZnSO_4$, but with no yeast extract, was tested in comparison with the Basic Medium. Under the conditions of the test, using *P. notatum* strain No. 4, the yields of penicillin were lower than with the Basic Medium.

Dunayer, Buxbaum and Knobloch (7) reported excellent results from growing *P. notatum* on tryptose-phosphate broth in a synthetic solution. In tests with a similar medium prepared with materials available the yields of penicillin were high, comparing very favorably with the Basic Medium.

Submerged Culture Method: Selected strains of *P. notatum* which are adapted to growth below the surface in aerated and agitated culture solutions are required for the submerged culture method since the strains used for surface culture do not grow well in submerged culture. As mentioned before, the submerged-culture method is an improvement over the surface method for the commercial production of penicillin. A culture of *P. notatum* No. 832 was obtained from the Northern Regional Research Laboratories* for comparative laboratory studies of surface and submerged culture.

Flasks, prescription bottles, and Baxter bottles (one liter capacity) were equipped with cotton filters in glass tubes for aeration, prior to the sterilization of the media. Media prepared according to the formula of the Basic Medium were used for the submerged-culture method. Comparison was made of the titer of culture solutions from Strain 832 in submerged culture and in surface culture, and with Strain No. 4 in surface culture. In surface culture of the two strains, both the culture solutions when tested after 10 days incubation by the Oxford cylinder method against *S. aureus* formed inhibition zones 33 mm. in diameter. Inhibition zones of approximately the same size were observed when the culture solution of Strain 832 grown by the submerged method was tested.

The only Hawaiian strain of Penicillium species (Strain No. 849) which was found to inhibit the growth of *S. aureus* was isolated from an over-ripe banana. It compared favorably in potency with Strain No. 4 when grown in surface culture. It was found that this Strain 849 also grew well in submerged culture and it was tested in comparison with Strain No. 832. In the only trial conducted Strain 849 produced a maximum titer of 15 units per cc. in 5 days as compared with 10 units per cc. in 10 days for strain No. 832. Fig. 11 shows inhibition zones formed by culture solutions of these two strains tested directly and in 1–10 dilutions.

The penicillin culture solutions may be filtered aseptically and concentrated by freezing and fractional meltings. The last fraction to freeze and the first to melt contains the highest concentration of penicillin (Fig. 12). The filtered solutions may also be concentrated by evaporation under partial vacuum at 40° C. to 50° C. The freezing method for concentrating the solutions was found to be superior to the evaporation method in the laboratory studies conducted. In either case the concen-

* Courtesy of Dr. Kenneth B. Raper.

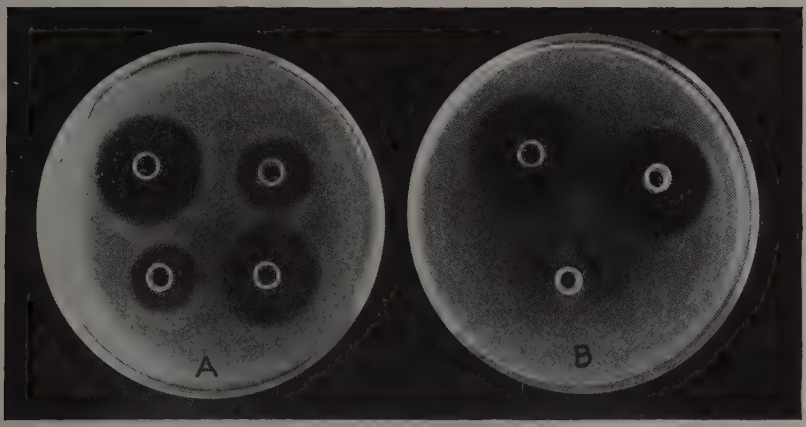

Fig. 11. *A*—Inhibition zones on plate of *S. aureus* formed by culture solutions, submerged method, of *P. notatum* No. 832 and *Penicillium* No. 849 (Hawaii). At top, left, *P. notatum* No. 832 after 10 days incubation (undiluted); at right, diluted 1 to 10. Below, *Penicillium* No. 849, after 5 days incubation; at left, diluted 1 to 10; at right, undiluted.

B—Inhibition zones formed on plate of *S. aureus* by culture solutions, surface method, of *P. notatum* No. 832 at left, and strain No. 4, at right; control test, below.

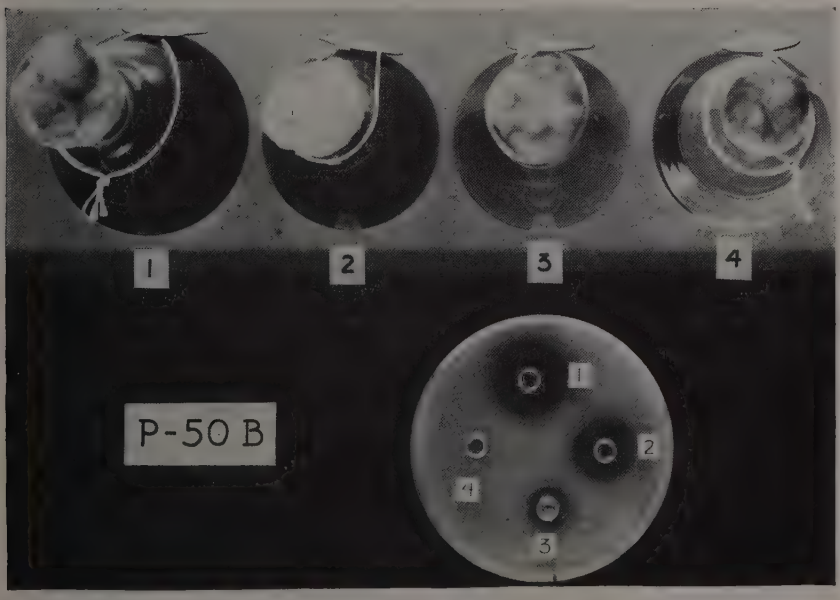

Fig. 12. Flasks of crude penicillin solutions separated by freezing and fractionally melting into solutions of decreasing concentrations of penicillin from the first fraction to the fourth as shown by their respective Oxford ring tests. A pronounced difference of color of these liquids exists: 1—dark amber; 2—light amber; 3—vivid yellow; and 4—light yellow.

trated solutions may be refrigerated and held for several weeks during which time they may be used for topical therapy.

A promising method for holding and shipping concentrated solutions, according to the results of a few preliminary tests, is by freezing the solutions which may later be melted when desired. All penicillin solutions held under refrigeration should be periodically tested for their potency.

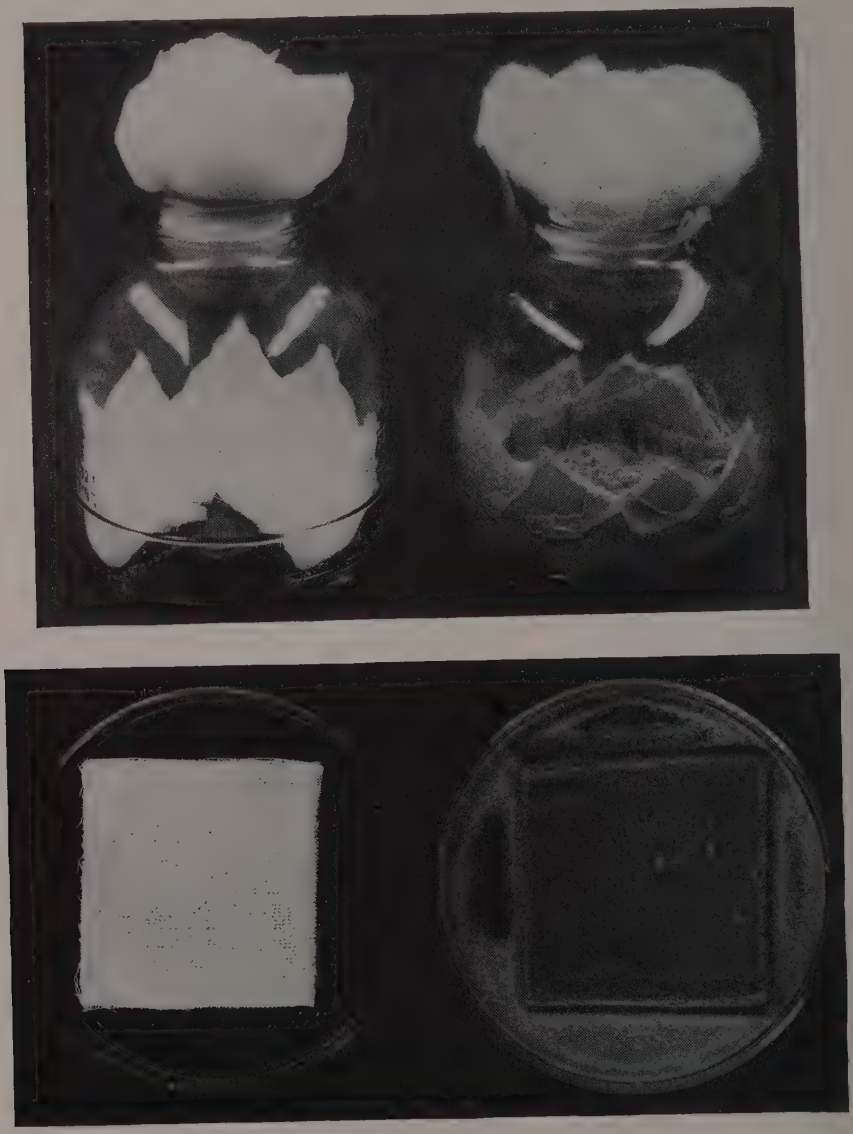

Fig. 13. Gauze squares on which the mold is grown for surgical dressings arranged by tens in Baxter bottles, or placed singly in Petri dishes. Only five pads were placed in the bottle on the left to facilitate showing their arrangement.

Inoculated Gauze Surgical Dressings: Independently of and almost simultaneously with Robinson and Wallace (17) one of the writers (C.W.C.) prepared gauze surgical dressings moistened with Czapek-Dox modified culture solution inoculated with *P. notatum* as a natural step following the successful use of mats of mycelium in topical application. Subsequently similar dressings were prepared using the formula they had suggested, substituting soluble starch for cornstarch. The titer of the liquid in these preparations was somewhat less than with our Basic Medium and required longer to reach a maximum. The yield of penicillin with the medium of Robinson and Wallace was only slightly improved by supplementing it with the inorganic salts of the Czapek-Dox solution, but no zinc. We have not found any medium superior to the Basic Medium for preparing inoculated gauze dressings.

Inoculated dressings were first prepared by placing one eight-ply gauze square in individual standard-sized Petri dishes. For the greater convenience of the physicians, especially the medical officers on board naval vessels and in army camps, a method was developed (by D.M.W.) whereby 10 standardized gauze squares (2¼″ by 2¼″ and eight layers in thickness) were arranged in a diagonally overlapping shingle fashion in the bottoms of 500 ml. Baxter bottles (wide-mouth, Fig. 13), an arrangement which exposed a maximum surface of each dressing for the growth of the mold. After the pads were autoclaved sterile Czapek-Dox Basic Medium was inoculated with a spore suspension of *P. notatum* and placed on the pads. There exists an optimum ratio between the surface area to volume of liquid on these pads (as well as in liquid cultures) and it was found that greatest speed of growth of the mold resulted when 15 ml. of the inoculated medium was pipetted onto the single pads in the Petri dishes and 100 ml. onto the 10 pads in the Baxter bottles. These dressings were ready for use in four (sometimes five) days and have been observed to retain their potency for more than 100 days under refrigeration (8°–12° C.). Such dressings have been in constant demand both by civilian and service physicians. They offer a convenient means of preparing penicillin at sea and in army camps for topical use. Upon request a number of service physicians and their technicians have been supplied with cultures of *P. notatum* and have been instructed in the preparation and use of these inoculated gauze dressings.

Measuring Penicillin in Solution—Method of Assay:

Two methods of assay are commonly used to determine the bacteriostatic or bactericidal properties of solutions: the serial dilution method in which various amounts of the solution are added to standard amounts of liquid culture media inoculated with the test organism, and the cup-plate method in which the solution is superimposed on agar plates inoculated with the test organism. We have used the modification of the cup-plate method devised by Heatley (1) in which cylinders of standard proportions are set on the surface of agar plates and then filled with the liquid to be tested.

Cylinders cut from plastic tubing were found practicable though glass and stainless steel cylinders were also employed. The plastic cylinders conformed to the dimensions suggested by Abraham, Chain *et al* (1): 9.6 mm. long, 5.1 mm. inside diameter and 7.2 mm. outside diameter; the ends were ground smooth and one end of each was bevelled either on the inside or the outside to facilitate penetration of the agar to form a water- and bacteria-tight seal (Fig. 8).

Poured plates of the test strain of *Staphylococcus aureus* were prepared and the medium (No. 2) allowed to solidify. One to four sterile cylinders were then set in the medium, equidistant when more than one were used per plate, and filled with the solution to be tested by means of sterile pipettes. The plates were then incubated at 37° C. for 16 to 24 hours though inhibition zones were often well defined in less than 8 hours. Unglazed clay Petri dish covers were found most satisfactory for preventing the condensation of moisture on the lids which often falls on the surface of the agar and obscures the results.

At the end of the incubation period most of the liquid in the cylinders had disappeared, and each cylinder which had contained bacteriostatic liquid was surrounded by a circular clear zone where the test organism had not grown. The diameter of this inhibition zone is a function of the bacteriostatic efficiency of the liquid (Figs. 8 and 14). Abraham, Chain *et al* (1) prepared a standard sample—a solution of penicillin in dilute phosphate buffer which was saturated with ether and kept in the refrigerator—for reference to evaluate new solutions. This standard solution produced inhibition zones with *S. aureus* averaging 24 mm. in diameter. They established what has become known as the Oxford unit, sometimes referred to as the Florey unit, which is "... that amount of penicillin which when dissolved in 1 c.cm. of water gives the same inhibition as this standard." The Oxford unit of penicillin has also been defined as "That amount of penicillin which when dissolved in 50 ml. of meat extract broth just inhibits completely the growth of *S. aureus* at a dilution of 1:50,000."

Medium No. 2 was disposed in tubes, 10 ml. for stock cultures, and 15 ml. for use in standard-sized Petri dishes. As required for test plates the medium was melted, cooled to 42° C., inoculated with young cultures of *S. aureus,* and the plates poured.

Since we were concerned in furnishing solutions containing an effective concentration of penicillin for external use rather than solutions containing stipulated unit concentrations carefully computed from a standard solution of known Oxford unit

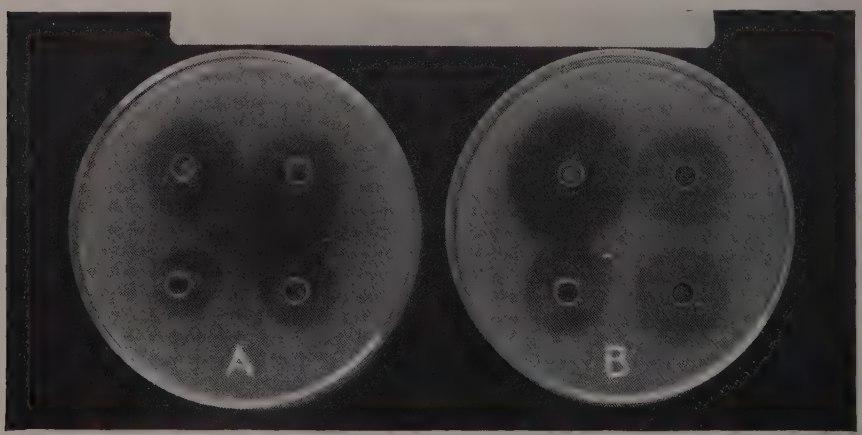

Fig. 14. Assay of culture solution from inoculated dressings with local (837), *A*, and Oxford strains, *B*, of *S. aureus.* Upper left (*A* and *B*), direct test; and clockwise, dilutions, 1–5, 1–10, and 1–20, respectively.

concentration per mg., it has been sufficient to estimate the unit value and supply only solutions which contained at least 4 units per ml. Some preparations have had a concentration well over 5 units per ml. as indicated by inhibition zones of 40 mm. with *S. aureus;* for a more accurate estimate of the unit value of such a solution, tests in a series of dilutions are required since 40 mm. is near the maximum diameter of inhibition zones regardless of the unit concentration (Fig. 14).

Topical Penicillin Therapy:

Approximately a hundred physicians in Hawaii are using the unconcentrated penicillin solutions and gauze dressings with outstanding success in treating specific infections. Clinical cases treated with these penicillin preparations greatly exceed in number those selected for publication by Larsen (15), Johnson (14) and Agmar (2); some additional cases are reported by Larsen in a separate paper accompanying this article. Cultures of *P. notatum* and instructions for preparing active solutions and surgical dressings were given to many medical officers of the armed forces as a result of which penicillin preparations are being made aboard naval ships which pass through Honolulu on their way to combat areas as well as ashore by various other medical units. As an example of this type of service it may be mentioned that cultures of the mold and instructions were given to Dr. A. R. Agmar (Lieut. Comdr., M.C., U.S.N.R.) who currently prepares about 40 bottles containing 400 inoculated dressings per week for use in his and other Naval Medical Units. In studies submitted for publication elsewhere Agmar reports successful and often dramatic results in 14 cases selected from many observed. The cases treated include extensive carbuncles, boils and furunculosis, cellulitis, chronic skin ulcers, impetigo, infected pilonidal cysts and two stubborn cases of sycosis barbae. His associates have met with similar success in treating conjunctivitis, corneal ulcers and otitis externa.

Summary

1. The discovery, properties, and production of penicillin and its application in the field of medicine are reviewed.

2. The prevailing temperature in Hawaii was found to be highly conducive to the growth of *Penicillium notatum.*

3. Stock cultures of *P. notatum* and subcultures made therefrom from which spores were obtained for inoculation studies were maintained on a nutrient agar medium. This medium (No. 1) proved highly satisfactory for maintaining the penicillin-producing properties of the fungus.

4. Different types of containers were used for culturing *P. notatum* on liquid media. The 32-ounce prescription bottle was finally adopted as the most desirable type available for growing the mold.

5. *P. notatum* was grown on a number of liquid media designed to increase the yield of penicillin as determined by the Oxford ring test. One of these (No. 4) was found to be superior to the other media tested and was used as the "Basic Medium" in all later experiments and in the production of penicillin solutions and gauze dressings for topical therapy.

6. One local and one imported commercial strain of *P. notatum* were cultivated by the submerged-culture method and tested for penicillin unitage. Although this improved method for commercial production was not used extensively the concen-

trations of penicillin in culture solutions compared favorably with those from the surface-culture method.

7. Filtered culture solutions were concentrated for refrigerated storage by evaporation at 40° C.–50° C. in partial vacuum; also by freezing and fractionating the solution into 3 or 4 portions as the ice slowly melted. The last liquid fraction to freeze and the first to melt contained penicillin in the highest concentrations.

8. Methods developed for preparing inoculated surgical gauze dressings are described. To conserve Petri dishes and for the greater convenience of the physician a new technique is described wherein 10 standardized gauze dressings in a 500 cc. Baxter bottle are inoculated. After 4 to 6 days growth the dressings are ready for use and retain their potency for 3 months or more if stored at 6°–10° C.

9. The two methods commonly used to determine the bacteriostatic or bactericidal properties of solutions are described. The penicillin in the various solutions discussed in these investigations was measured by the modified cup-plate method of Abraham, Chain *et al* substituting plastic cylinders for glass.

10. References are given to topical penicillin therapy and to clinical cases treated with penicillin solutions and gauze dressings by physicians in Hawaii.

Acknowledgement

The moral and financial support of the Trustees and the Experiment Station Committee of the Hawaiian Sugar Planters' Association, and the foresight of the Director of this Station, made possible this contribution to the humanitarian relations of the Association not only to its employees but to the community in general. The penicillin investigation and the development of a practical service to the plantations and to the community would not have been functionally complete without the constructive cooperation of Dr. Nils P. Larsen.

It is believed that the project has contributed materially to labor turnout, and to the relief of hospital congestion, and that it has benefited the health and morale of service men as well as civilians. The results, often dramatic, reported by physicians in the majority of cases of stubborn infections wherein penicillin therapy was indicated justify this essay into the field of mycology applied to chemotherapy. The urgent demand for penicillin preparations not otherwise obtainable has been met without discrimination and without cost to the community. We greatly appreciate the continued interest of physicians in the Territory in these studies and their reports on the success of topical penicillin therapy.

Appreciation of the cooperation of the following staff members also is gratefully acknowledged: F. Ray Van Brocklin for collaboration in chemistry and determining and adjusting the hydrogen-ion concentrations of culture media; to W. Twigg-Smith and Y. Yamamoto for the illustrations, and to staff members and all others who have assisted in the studies.

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Clinical Studies With Crude Penicillin

By Nils P. Larsen, M.D.

The preparation of penicillin solutions and surgical gauze dressings at the Experiment Station, H.S.P.A., and the training of a technician from a plantation hospital on each island in making penicillin materials so they would be available to plantation physicians are discussed. The results of ten clinical cases wherein crude penicillin was used for topical therapy are presented. Summaries of individual reports by doctors using crude penicillin on specific cases are given. The merits of crude penicillin over sulfa drugs for treating certain infections are pointed out. It is suggested that crude penicillin has a definite place in topical therapy.

The mold *Penicillium notatum,* which produces the substance penicillin reported by many observers to be very effective in treating wounds, was obtained as authentic cultures in the latter part of 1943 and grown at the Experiment Station of the Hawaiian Sugar Planters' Association. The three men working on this project, J. P. Martin, C. W. Carpenter and D. M. Weller, were all experienced in research.

One of four strains of *P. notatum* which they obtained was found to inhibit *Staphylococcus aureus* to a greater extent than the other three. With certain modifications of the medium on which the fungus was grown they were able to increase the penicillin content of the solution. The solution was assayed by the Oxford ring test, *i.e.,* a small quantity (standardized) of the solution was placed in a small standardized container on the surface of an agar Petri dish heavily seeded with *S. aureus.* The plate was then allowed to incubate for 16 hours. At the end of this time the width of the clear zone (free of bacterial colonies) around the container was measured in millimeters. The stronger the penicillin solution, the wider was the clear ring or inhibition zone. The crude penicillin solution is a clear, deep amber liquid. If kept on ice it remains effective for a month or more.

Sterile gauze pads placed in jars and moistened with the culture solution were also seeded directly with spores of *P. notatum.* When the fungus cultures were four to five days old and became green, they were placed in the icebox. These soaked gauze dressings with the fungus growing on them were used directly on infected wounds. Such dressings were found to have a higher concentration of penicillin than the crude culture solutions. The patient who used the gauze was usually also given a bottle of crude penicillin solution and told to saturate the dressing every three hours.

Clinical Studies

The solutions of penicillin and the inoculated gauze dressings were prepared in the Experiment Station, H.S.P.A. laboratories. Each batch was checked against *S. aureus* so that the degree of potency was known. However, many other chemicals show a striking potency when similarly tested, but fail to give comparable clinical results. It was therefore important to determine whether the penicillin culture preparations would produce striking effects on patients.

Dr. Harold Johnson* treated fifty cases of impetigo and allied diseases of the skin, and reported consistently good results with crude penicillin dressings. We selected a series of fifty cases† which had been carefully observed by a group of doctors. The majority of these cases also showed excellent response to the penicillin.

A technician from a plantation hospital laboratory on each island came to the H.S.P.A. laboratories in Honolulu to learn exactly how to cultivate *P. notatum* and to prepare the solutions and inoculated dressings. All the plantation doctors were then notified that the penicillin solution was available. The doctors used it on far more cases than were reported, for instance, we recently received from one laboratory the following note:

"We've been having unusually good results with our cultures.... (Dr. W. also has been using it quite consistently and is having good results.)"

From that island we received no specific reports, hence no cases from that island were included in our series of fifty. This indicates it was used far more widely than suggested by the number in our series.

The following cases are selected from the fifty as representative of type results, and indicating how this solution works.

Case 1: A surface infection of the ankle had been treated by sulfa drugs without relief. The infection was getting out of hand, and a goodly portion of the ankle when first seen gave the appearance of a ploughed field, with pus pools in the furrows. The patient had previously had a trychophytin infection in this area, but after he made a trip south it became infected with staphylococci. Two of the inoculated pads were placed on the lesion on the first day. The patient was told to soak the gauze every three hours with the crude penicillin solution. He returned the next day and the wound still looked rather ugly, but there apparently was some decrease in pus. New pads were applied every twenty-four hours. After forty-eight hours the wound was markedly improved and there was almost no pus. After seventy-two hours we considered the wound healed. Although there was still evidence of trychophytin infection, the purulent infection had cleared completely. This indicates rather strikingly that: (1) a surface infection can be treated ideally by this penicillin solution; (2) this solution does not kill all organisms or help all infections (however, in the presence of a trychophytin infection which was not influenced, the purulent infection healed completely); (3) this solution penetrates pus and dead tissue, which apparently often keep the sulfa drugs from being effective; and (4) when the organism producing the infection is affected by the solution, the results are dramatic. We concluded after seeing a good many cases that if the results are not dramatic, then the penicillin is probably not affecting that particular infection.

Case 2: An individual with diffuse impetigo on both thighs. The plantation doctor in this case wanted to compare penicillin with sulfa drugs. He therefore applied sulfa ointment to one thigh and crude penicillin solution to the other. By the fourth day both lesions were entirely healed. In this case this particular infection could probably have been healed just as well with sulfa drugs. However, a

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* His first report is published in the July 1944 issue of The Archives of Dermatology and Syphilology, Vol. 50, pp. 1–5.

† First group reported in the October 1944 issue of The Hawaii Medical Journal.

good many patients are sensitive to sulfa drugs. A severe dermatitis can be produced from local application. This type of reaction from crude penicillin we have seen only once but this patient was also very sensitive to all the sulfa drugs. This case illustrates another reason why the crude penicillin has a place, *i.e.*, although the wonder drug of the past few years (the sulfa drug) may inhibit a particular infection just as well as crude penicillin, nevertheless the crude penicillin has the place of preference, since there is little chance of a severe skin reaction.

Case 3: A purulent infection in a young girl involving both hands. This girl had a sensitive so-called atopic dermatitis of both hands. She developed a very severe purulent infection with blisters and pus pockets in scattered areas on each one of the fingers and in the palms and on the backs of both hands. She was completely incapacitated, and could not open her hands. Since she was a sensitivity type we were very fearful of using the sulfa drugs. We therefore applied penicillin. In forty-eight hours practically all the pus pockets had cleared. By seventy-two hours we could discharge the girl as cured. In this case we did not dare to try sulfa drugs.

Case 4: A woman with diabetes, in poor general condition and with a huge carbuncle on her back. This carbuncle was excised, leaving a crater at least six by eight inches in diameter. It was oozing pus and surrounded by dead tissue. Rubber tubes with multiple openings were placed in the crater. The ends of the tubes were left clamped outside of the dressings. Every three hours the nurse injected 10 cc. of the crude penicillin solution into the free end of the tubes. Although this case did not show as dramatic improvement as some, the tissue itself began fairly rapidly to look much healthier. The amount of pus decreased. With control of her diabetes and with the open drainage and with the help of the crude penicillin solution, she did slowly make a complete recovery.

Case 5: Patient had a large infected crater ulcer of the leg. This wound had been open and draining pus since 1936. The crude penicillin was applied every three hours. The wound culture showed staphylococci, streptococci, as well as other organisms. The wound did very well for a period of time and almost healed. Then it began to do badly again. When it was again cultured, however, it was found to contain none of the staphylococci or streptococci, but only the penicillin-resistant organisms. This case illustrates well that penicillin does not affect all organisms. It will be beneficial only in those cases where the organism producing the lesion is or can be affected by the penicillin. Other organisms can produce the same type of ugly lesion, and these organisms may be influenced in no way by the penicillin.

Case 6: Infant with diffuse impetiginous lesions. In this case the mother was shown how to apply the penicillin every three hours. Since babies are unable to cooperate, it might be dangerous to use mercury or other poisonous drugs. This baby was treated freely with penicillin, and in twenty-four hours the lesions were practically well. Penicillin is doubly effective because it penetrates the scabs, often difficult to remove in young children.

Dr. Donald Marshall (a pediatrician) reports that he has used crude penicillin on a number of cases of impetigo in children, in some instances in individuals sensitive to sulfonamide powder. In all patients the infection was rapidly cleared, usu-

ally in about twenty-four hours. However, he makes it a rule first thoroughly to cleanse the lesion and remove all crusts.

Case 7: Patient aged 15 with a draining sinus from the spine and hip. This boy had had a draining sinus for a period of many months. Pus could be obtained from it by pressure at any time. A rubber catheter was inserted deep into the sinus, leaving the open clamped end protruding above the dressing. Every three hours 10 cc. of crude penicillin were injected into this open end. After three days the boy was better; after a week he was able to get up and about; and in two weeks left the hospital with no pus coming from the sinus. The staphylococci which had been cultured at first soon disappeared. This case illustrates the use of the crude drug in treatment of deep sinus tracts.

Case 8: Case of a janitor with diffuse staphylococcic skin infection of both feet. There were numerous pussy craters where blisters had been rubbed off. The whole area was swollen, red and tender. The skin was soggy and cyanotic. Penicillin pads were applied. When seen twenty-four hours later the swelling and redness of one foot had cleared. There was still a deep fissure between the large and second toes. The left foot was still swollen and showed some craters, but the patient said the soreness was gone. He changed pads twice a day. In seventy-two hours both feet were well.

Case 9: Infected incision following a Caesarean in a patient 45 years old with diabetes. The infected incision had been treated for forty-two days, but was still oozing pus. After first penicillin pad there was no change. After second pad purulent discharge seemed to increase. After third pad the discharge diminished. After seventh pad the discharge stopped. Incision went on to full healing.

Case 10: Patient aged 43 had suffered from varicose veins since birth of her first child in 1922. First varicose ulcer of the right ankle developed in 1928. The longest period since that time that it had been completely closed was six months. Prior to the use of penicillin pads the ulcer had been open, weepy and pussy for a period of two years. Application of penicillin pads was begun in May of 1944, at which time there was a large crater ulcer with much pus. Penicillin pads were put on once a day, and solution put on every three hours. The pain from the wound seemed to be relieved with the application of the pads. By September it was completely closed, but a month later it opened again, and for a month no benefit was noted from penicillin pads. The culture solution of a yeast-like organism (*Torulospora utilis* var. *major*), found effective *in vitro* against gram negative bacilli as well as gram positive bacteria by Carpenter*, was applied. In twenty-four hours the discharge had practically stopped and the wound looked markedly improved. This case illustrates the use of penicillin in an area where poor nourishment of tissue almost certainly will produce breakdowns and ulcer formation, but the penicillin inhibits the growth of the bacteria and therefore keeps the ulcer fairly well under control. It also suggests the use of the crude yeast solution when the penicillin does not affect the infecting bacteria. It is known for instance, that colon bacilli are not affected by penicillin but are by the yeast solution.

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* Carpenter, C. W., 1945. Antibacterial properties of yeasts, Fusarium sp., onion and garlic. The Hawaiian Planters' Record, 49: 41–67.

To illustrate the doctors' reactions to cases treated, I quote from different plantation doctors' summaries of individual reports of various types of surface or open infections:

"Responded remarkably well."
"Not a complete cure."
"Improved after first dressing, well after second."
"No pus after first application. Redness decreased."
"Immediate improvement."
"Healed over completely."
"Improved in twenty-four hours."
"Discharge diminished."
"Dramatic improvement after the second dressing."
"Excellent results in forty-eight hours."
"General condition good. No pus."
"Cleared but maybe it would have cleared anyway."
"Cleared in twenty-four hours."
"Good; about in same time as sulfa ointment side."
"Infection markedly improved."
"Excellent."
"Cured."
"Cured."
"Healed in eight days."
"Temperature to normal."
"Discharge decreased."
"No response."
"Rapid improvement."
"Pus and redness getting much less."
"After twenty-four hours marked improvement. Complete healing after six days."
"Marked improvement in twenty-four hours."
"Small amount of pus each dressing; none after one week."
"Markedly benefited."
"Wound quickly became clean."
"Results truly spectacular."
"Cleared rapidly. Apparently excellent response."
"Ulcer covered with necrotic membrane with pus underneath. Cleared up next day."
"Sinus appeared clean after third day."
"Redness and irritation receded. Pus formation lessened."

Discussion

If the organism was one that was sensitive to penicillin, the effects were dramatic. If there was not a dramatic change in the wound or the infection in forty-eight hours, suspicions arose that the causative organism was not sensitive to penicillin. For impetigo it was particularly effective. Even without removing the crusts, the penicillin has the ability to penetrate dead tissue and penetrate the crusts and affect the organisms underneath.

If sulfathiazole can be applied directly and the wound properly treated, it is probably just as effective in this condition as penicillin. However, in a hot climate the use of ointment tends to keep the skin moist and soggy, and if with the high temperature goes a high humidity, then the skin underneath the ointment will become less bacteria resistant. In such cases crude penicillin has a definite advantage over any type of ointment. Secondly, since we occasionally get a rather severe reaction from locally applied sulfa drugs, the latter are in such cases contra-indicated. A sensitivity reaction to penicillin is extremely rare. The crude penicillin in some wounds occasionally produces slight discomfort for about fifteen minutes, yet it can be used in large open wounds without fear of toxic absorption.

Since pure penicillin is still not available in sufficient quantities to treat all minor skin infections, and since our climate is ideal for growing *P. notatum* at room temperature and it can therefore be readily grown in large quantities, we believe that crude penicillin has a definite place in therapy here.

Penicillium cultures are now being grown on each of the other islands, available for any doctor for any type of open lesion. The cultures, however, must be watched to make sure that the effective *P. notatum* is kept growing. Occasionally there should be a check for potency.

Summary

We feel the experiments of the Experiment Station, H.S.P.A., conducted to help the many employees of the sugar industry suffering from rather annoying and long continued surface or open infections, have taught a lesson which could well be applied on a wider scale. Wherever there is a hospital laboratory, penicillium culture solutions and inoculated gauze dressings can be prepared, and penicillin in these forms should be available for every person needing it. Clinical studies of a new self-propagating drug which can be prepared in any good laboratory are reported. It is particularly useful in hot humid climates where purulent skin infections are common. Its effect on such infections is more striking than any drug previously used. Reports are given on ten type cases out of one hundred studied. The work was made possible by the untiring efforts of C. W. Carpenter and D. M. Weller and the cooperation of J. P. Martin of the H.S.P.A. Experiment Station.

A Preliminary Report on the Production and Use of Crude Penicillin Solution in a Naval Dispensary

By Albert R. Agmar, Lt. Comdr. MC-V(S), USNR.

The technique developed at the Experiment Station, H.S.P.A., for the preparation of active solutions of penicillin and inoculated gauze surgical dressings was applied in a naval dispensary. These materials have proved dramatically effective for such minor infections as boils, carbuncles, secondary epidermophytosis of the feet, eye and ear infections, chronic leg ulcers, infected cyst teratoma, etc., for which purified penicillin was not obtainable. In these cases the usual period of hospitalization was reduced materially, an important factor in activities with limited bed capacity and large transient case load. Fourteen selected cases treated successfully with the active preparations are described. This project has been officially authorized for expansion to establish a source of supply of penicillin solutions and inoculated surgical dressings, and possibly other similar antibacterial preparations for the Fourteenth Naval District.

The penicillin allotted to naval dispensaries is designated for certain specific purposes so that many patients with types of minor localized infections that would be benefited by its use are, up to the present time, unable to be treated with it.

Fleming (5) suggested that filtrates of broth cultures of *Penicillium notatum* might be efficient antiseptics for topical applications to areas infected with penicillin-sensitive microbes. It has been noted by many workers that topical application of solutions of pure penicillin are very effective in the treatment of localized infection. Florey and Florey (6) used such solutions with success in the treatment of infections of the eyes, mastoids, etc.

In Hawaii, where pure penicillin for civilian use was practically unobtainable until very recently, Larsen (9) treated 23 cases with culture solutions of *P. notatum* or surgical dressings inoculated with this mold. In a current paper (10) he cites ten representative cases selected from an additional fifty cases treated. Johnson (8) reported the successful use of such penicillin preparations in extensive cases of impetigo which had proved resistant to the usual mercury and sulfathiazole treatments. He stated, "Its action on staphylococcus makes it especially suitable for use in skin diseases."

The inoculated gauze dressings were developed in 1943 by Carpenter (3) independently of and almost simultaneously with the similar dressings described by Robinson and Wallace (12). The solutions and inoculated dressings used by Larsen (9, 10) and the major portion of those used by Johnson (8) as well as those used at first by the plantation hospitals of the Hawaiian Sugar Planters' Association were prepared at the Experiment Station of that Association by Carpenter, Weller and Martin (3) under the supervision of Dr. Nils P. Larsen. Several hospitals on the islands of Hawaii, Maui and Kauai currently prepare their own penicillin materials for more efficient distribution. Carpenter, Weller and Martin have developed a simple, efficient method of production that can be carried out with

a minimum of equipment by any technician after a few hours of instruction. It could be carried out rapidly in any naval medical activity once the necessary chemicals, bottles, and a pure culture of the mold *P. notatum* known to produce penicillin are obtained.

The Experiment Station, H.S.P.A., has supplied large quantities of culture solution and inoculated gauze dressings to doctors and hospitals in the Hawaiian Islands for over a year with consistently excellent reports of their successful use. This wide use of crude penicillin solution, applied locally, has shown that it combines several very desirable qualities, among which may be mentioned the following:

1. Low toxicity to tissue cells.
2. Highly potent bacteriostatic action.
3. It is not inhibited by pus, peptone, blood or autolysates.
4. It is confined to the site of infection.
5. There is a high local concentration where needed.
6. It prevents the spread of infection.
7. It is fast acting.
8. Susceptible organisms do not seem to become "penicillin fast."

At the medical station where this work was conducted, the use of penicillin gauze and crude penicillin solution has been found to reduce considerably the usual length of hospitalization in such local infections as boils, carbuncles, secondary infections in epidermophytosis of the feet, external auditory canal infection, otitis media, eye infections, infected cyst teratoma, chronic leg ulcers, etc. This time saved is an important factor in an activity which has a relatively small bed capacity and a large transient case load.

The use of sulfonamides has been an aid in cutting down the time lost by such infections but they are frequently found ineffective in certain cases and often cause toxic reactions. Crude penicillin solution so far in our experience has not caused any reactions and works more rapidly than the sulfonamides, with no case apparently becoming "penicillin fast."

We have used the methods developed by Carpenter, Weller and Martin (3) for the production of penicillin preparations, which may briefly be outlined as follows: The culture medium is a modified Czapek-Dox medium to which 0.001 gram of zinc sulfate and 10 grams of yeast extract (Difco) are added per liter. These supplements apparently favored the rapid growth of *P. notatum* and a more rapid production of penicillin. The complete formula of the medium is as follows:

KH_2PO_4	1.0 Grams
KCl	0.5 "
$MgSO_4—7H_2O$	0.5 "
$NaNO_3$	3.0 "
$ZnSO_4—7H_2O$	0.001 "
$FeSO_4—7H_2O$	0.01 "
Glucose (candy-makers)	40.0 "
or	
Dextrose C.P.	14.8 "
Yeast extract (Difco) 1%	10.0 "
Distilled water q.s.	1000.0 cc.

Adjust to pH 6.5 with N/10 NaOH or with sodium citrate.

If candy-makers glucose is used, dissolve the forty grams in hot distilled water (about 100 cc.) and pour the solution into a one-liter volumetric flask, then add the salts in the order listed. Then add the yeast extract and distilled water sufficient to make one liter. The pH is adjusted after the ingredients are thoroughly distributed in the medium.

Carpenter found that the pH of the medium could be adjusted with practical accuracy by determining the amount of sodium citrate required to adjust one liter to pH 6.5–6.8, and to use this amount of sodium citrate, or a multiple of it, for subsequent batches of media wherein the same lots of materials were used. This method is convenient in laboratories where colorimeters or potentiometers are not available. We have used this method and checked the pH value roughly with nitrazine test paper and standard color charts. We have also used standard-test color solutions in a La Motte colorimeter with chlorophenol red and bromthymol blue as indicators. As occasion required, F. R. Van Brocklin, Associate Chemist, Experiment Station, H.S.P.A., has kindly determined the pH values of our culture media and culture solutions with a potentiometer.

In preparing gauze dressings for inoculation with *P. notatum* we have adopted a technique devised by Weller (3). Gauze squares 2¼ by 2¼ inches are cut from eight-ply 20 by 12 gauze. Ten of these dressings are placed in an overlapping shingle arrangement in a 500-cc. Baxter intravenous glucose bottle, one-half of each dressing resting against the side and the other half against the bottom of the bottle, with

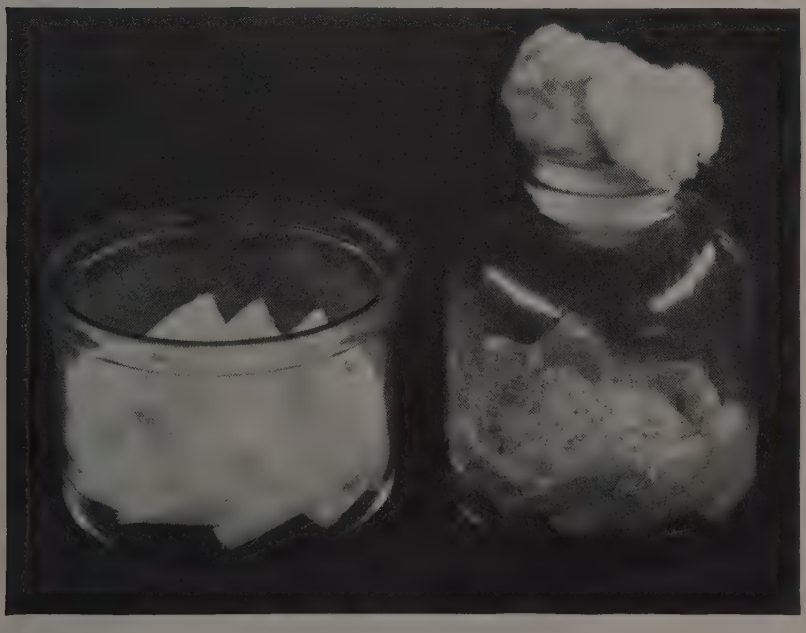

Fig. 1. Arrangement of ten gauze dressings in Baxter bottle. At left, open jar showing more clearly the overlapping-shingle pattern, exposing a portion of the surface of each pad. Dressings at right in Baxter bottle showing mature growth of *Penicillium notatum* with the dressings ready for use.

a point of each projecting upward (Fig. 1). One hundred cc. of the medium are added to each bottle which is stoppered with a loose cotton plug. The bottles are then sterilized at fifteen pounds pressure for fifteen minutes and allowed to cool in the sterilizer to prevent breakage and contamination by any inrush of air if cooled too rapidly. When sufficiently cool each bottle is inoculated with two cc. of a spore mixture prepared by first adding enough sterile distilled water to a nutrient agar slant of a pure culture of *P. notatum* to just cover the mold growth. The age of this source culture for the spore suspension is important; best results follow when the culture used for inoculation is about four or five days old and not older than ten days; cultures reaching a suitable stage for inoculation may be held under refrigeration until needed. With sterile technique the spores are scraped from the slant culture with a platinum loop. The tube is flamed again and plugged and agitated for several minutes. The spore suspension is added to 25 cc. of sterile distilled water and mixed thoroughly by shaking to distribute the spores. After the two cc. of the spore suspension are added to the medium in each of the bottles by means of a sterile pipette, the bottles are agitated gently to distribute the spores over the pads without disarranging them.

The bottles of dressings are kept at room temperature until the mold becomes blue-green in color which indicates maximum penicillin formation; this usually takes place in four or five days in Hawaii. The liquid and the lower surface of the dressings are by this time golden yellow. The pads are then ready for use and must henceforth be refrigerated to maintain potency. The fluid in the bottles should be assayed at this time. Once having become yellow the fluid maintains potency for from three to ten weeks under refrigeration.

The individual dressings are removed as needed with sterile forceps and placed directly on the wound, mold side up. To prevent absorption of the fluid into the external dressing we place a piece of waxed paper over the penicillin impregnated gauze and apply any necessary dressing or bandage over it. The gauze pads must be kept moist at all times to be effective. They should be changed when dry or more penicillin solution may be added from time to time. A small amount of penicillin solution in a sterile Rx bottle may be given the patient with instructions to add it to his dressing when it becomes dry. This supply bottle should be refrigerated when not actually in use.

The Baxter bottles of Penicillium-inoculated gauze are especially useful in the Navy as they are easily transportable, the small amount of medium is held in the gauze, and the excess fluid is not enough to slop around or spill out. The bottles can be transported to other dispensaries and ships; refrigeration is unnecessary between the time of inoculation and the development of the blue-green coloration of the surface of the pads and the yellowing of their lower surface (a period of four or five days).

Crude penicillin solution is adaptive for irrigations and for moistening dressings. This is prepared in quantity by inoculating 250 cc. of sterile Czapek-Dox modified medium in flat 32-ounce Rx bottles with five cc. of spore suspension; after thoroughly mixing, the bottle is placed on its side and incubated at room temperature. In about five days a thick mat of the mold covers the surface of the medium. With maturity of the growth the mat turns from white to blue-green, and then to gray. The culture liquid should then be assayed biologically for potency. We use the

Oxford ring test (Figs. 2, 3) described by Abraham *et al* (1) for this purpose. Atkinson (2) compared several quantitative methods for the assay of penicillin.

In the Oxford ring test, a standard strain of *Staphylococcus aureus* is planted in a tube of liquified nutrient agar by means of a platinum loop, the temperature of the agar not exceeding 42° C. The medium is agitated to distribute the bacteria and is then poured into a sterile Petri dish and allowed to harden. Sterile Oxford cylinders (Fig. 3), variously called rings and cups, made of standard size, from glass tubing, or in our case from plastic tubing, and bevelled at one end, are placed on the

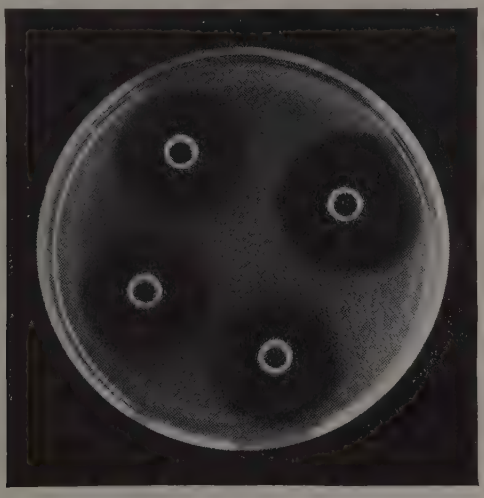

Fig. 2. Oxford ring test. Inhibition of *Staphylococcus aureus* by crude penicillin solutions. The diameter of the clear zone around the cylinders is a measure of bacteriostatic potency.

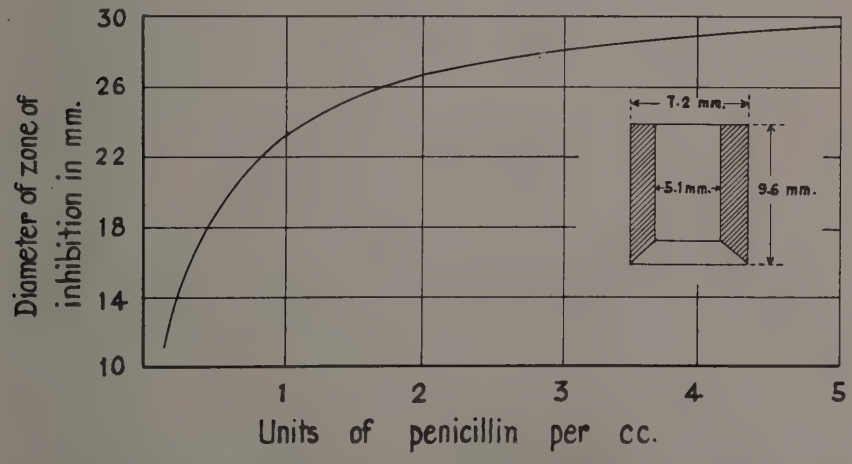

Fig. 3. Graph for estimating the number of units of penicillin per cc. of culture solution. Also median vertical section through assay cylinder with dimensions in millimeters. After Abraham and his associates (1).

plate inoculated with the staphylococcus. They are pushed lightly into the agar to seal the lower end. Usually four of the Oxford cylinders are put on each plate so that four tests can be run at one time.

Each cylinder is filled with a sample of the penicillin solution to be tested by means of a sterile pipette. The Petri dish is then covered and incubated for 16 to 24 hours at 37.5° C. The diameter of the clear inhibition zone around each cylinder indicates the unitage of the tested liquid (Fig. 2). This clear area is usually 25 to 30 mm. in diameter with potent culture solution. By diluting the solutions to be assayed with sterile distilled water 1–2 and 1–10 a more accurate estimation of unitage may be calculated. The average strength of potent crude solution is five units of penicillin per cc.

When the cultures of *P. notatum* in the 250-cc. bottles have reached maximum potency, usually in eight to ten days, the solution is filtered through sterile filter paper into sterile bottles and kept under refrigeration. This solution may be used to saturate gauze dressings or to irrigate sinus tracts or open cavities as in empyema, etc. For ear cases we use gauze wicks ½ by 2 inches placed in small, wide-mouthed, stoppered bottles, sterilized and saturated with crude penicillin solution.

T'ung (14) increased the potency of the culture solution under partial vacuum and low temperature. Carpenter (3) concentrated the culture solution by freezing and allowing the frozen solution to melt slowly at room temperature. The first and second meltings were collected separately, being differentiated by color tints. Weller (3) continued this study and separated the liquid into four fractions with decreasing unit value. The last fraction to freeze and the first to melt is darkest in color and contains the greatest concentration of penicillin. The lightest colored solution and the last to melt was found to be almost devoid of penicillin. However, the crude culture solution as originally produced is sufficiently potent for most cases.

In preparing penicillin culture solution and Penicillium-inoculated gauze dressings, and in the use of these materials the following suggestions are important:

1. Avoid contamination. Use sterile technique at all times. Contamination renders the solution inert very rapidly. A cloudy solution is usually a contaminated solution.
2. Be sure the Penicillium culture used is a pure culture of an authentic penicillin-producing strain of *P. notatum* from a reliable source. (Raper and Coghill (11) and Thom (13).)
3. Assay each batch biologically. (Foster and Woodruff (7).)
4. Culture and identify the bacteria causing infection if possible. The organisms should be known to be sensitive to penicillin prior to treatment.

It is planned, on the request of Rear Admiral Lucius W. Johnson, District Medical Officer of the Fourteenth Naval District, who investigated this method of production and use of crude penicillin solution, to produce enough of the dressings and solution to supply any interested naval activity or ship in this district and to train others in their production. We feel now that a source of a rather scarce and potent drug is readily available to anyone who may be willing to produce it wherever he may be.

The performance of penicillin has stimulated a world-wide search for similar substances of microbial origin and even the higher plants have not escaped scrutiny.

This search has already revealed a number of substances which may prove useful. Johnson (8) mentioned the then unpublished studies of Carpenter (4) with yeasts and the yeast-like organisms *Torulospora utilis* and its varieties. These organisms in varying degree inhibit not only *Staphylococcus aureus* but also the gram-negative *Bacillus coli* and *Bacillus pyocyaneus, in vitro*. Gauze dressings moistened with a suitable medium have been prepared and inoculated with *T. utilis* var. *major* in a manner similar to the Penicillium dressings described above. Dressings of this type as well as active solutions were used successfully by Johnson (8), Larsen (10, in Case No. 10) and in Case No. 12 cited below. In the few clinical trials thus far made these organisms and their products have appeared to be non-toxic in topical use and of promising value in clearing secondary infections.

Case No. 1: C. D., PhM. 3c, 9/27/44: Cellulitis of right foot, secondary to fungus infection, onset 9/26/44: redness, swelling, multiple small blisters and much itching. Temperature 100. Pulse 94. First seen and penicillin gauze applied 9/27/44. Sulfathiazole by mouth 75 grains daily for two days previous to entry. 9/29/44: No itching, area of redness much smaller; patient stated the dressing caused a warm, drawing sensation locally. 9/30/44: No redness, no itching and skin healed. Patient returned to duty.

Case No. 2: C. D. C., S2c: Infected lacerations, third and fourth fingers, right. Date onset 8/28/44. Deep lacerations, edema pain, redness, much pus. Had been treated with sulfathiazole dressings and sulfathiazole by mouth. Hot soaks. Temperature 99.6. Pulse 90 W.B.C. 15,400. Sulfonamides stopped. 9/25/44: Penicillin gauze applied after hot soak. 9/26/44: Wound improving, healing rapidly, no pus. 9/27/44: No pus, edema less, redness nearly gone, no pain. 9/28/44: Much improved, almost healed. 9/29/44: Laceration healed. Patient discharged to duty.

Case No. 3: R. S., S2c: Boil, anterior neck. Onset 9/20/44: Previous treatment—hot stupes. Appearance—1-inch diameter, very painful, red, hard, small pointing head. Opened and drained. Penicillin pads applied. 9/23/44: No redness, no pain. Very small amount of pus in crater. 9/24/44: No redness, no pain, no pus. Healing rapidly. 9/25/44: Practically healed over. Dry dressing. Patient discharged.

Case No. 4: O. H. E., F1c, 9/12/44: Chronic pus infection in old laceration. Anterior portion lower ⅓ right leg. Painful and red. Date onset 8/30/44: Previous treatment of large fish-hook-shaped laceration—hot stupes. Sulfathiazole locally. First penicillin pad 9/12/44, and sulfonamides stopped. 9/13/44: No pain. Patient states had warm sensation in wound for several hours after application of penicillin pad, and pain began to subside. Very small amount of pus noted. 9/14/44: Much improved. Dry dressing applied. 9/15/44: Dry dressing too irritating. Patient requested more penicillin pad treatment, which was done. 9/16/44: Improving. 9/17/44: Improving, clean. 9/18/44: Slight bleeding from healthy granulation tissue when pad removed. 9/19/44: Wound clean, healing rapidly, sulfathiazole applied. 9/20/44: Almost complete healing. Boric-unguent dressing. 9/21/44: Condition improved. Patient discharged.

Case No. 5: R. K., Sp(I)1c, 9/4/44: Markedly tender painful abscess between rectum and scrotum. Patient unable to walk or sit without severe pain. Date onset 9/1/44: Opened and drained 9/4/44. Penicillium gauze pad applied.

9/5/44: Pain very slight, no redness, no pus. 9/6/44: No pain, slight soreness to pressure, no pus. Ammoniated mercury applied. 9/7/44: Practically healed.

Case No. 6: E. T. L., F1c: Secondary infection, fungus, right thigh and groin. Onset of secondary infection 8/10/44. Previous treatment—warm potassium permanganate stupes, sulfathiazole dressing. Large, red, raw area with much serum and pus in right groin and thigh. Very tender. First penicillin pad applied 9/12/44, and sulfonamides stopped. 9/13/44: Wound clean, no pus, less red. Patient comfortable. Penicillin pads applied. 9/14/44: Secondary infection apparently cleared up, no pus, no exudate, no redness. Skin practically healed over. Fungus infection apparently present at edges of former irritated area.

Case No. 7: E. P., S1c, 9/1/44: Chronic infection anterior tibial region, left leg. Present about one year. Patient originally struck leg on rung of ladder. Infection set in and spread to area 3 inches long by 1 inch wide—indurated red, slight pus discharge and very painful. States he had had almost continuous pain for nearly a year. He had had bed rest, hot stupes, all kinds of ointments, etc. No varicosities noted. Penicillin pad applied. No other form of treatment used thereafter. 9/2/44: Some improvement—less pus. Patient states he experienced feeling of warmth in wound after application of pad and had his first relief from pain in one year. Penicillin pad applied. 9/3/44: Patient was treated by corpsman who started to apply a sulfathiazole ointment dressing, but patient refused it saying he wanted the doctor to see it and put on more of that "new stuff." The wound had decreased to approximately one-half its former size. Was clean and healthy granulated, tissue was present. Penicillin pad applied. 9/4/44: Improving. Penicillin pad applied. 9/5/44: Improving. Penicillin pad applied. 9/6/44: Improving. Penicillin pad applied. 9/7/44: Wound practically closed in. 9/8/44: Wound healed. Patient discharged to duty.

Case No. 8: P. R., PhoM1c, 9/4/44: Otitis externa, right. About five months duration. In and out of hospital several times but never completely healed. Entered this dispensary one week after discharge from hospital. Entire external canal acutely inflamed and swollen so as to almost completely obstruct the orifice. The slightest movement or pressure on the external ear caused severe pain. A penicillin saturated gauze wick was inserted into the ear and in between four and five hours the pain had subsided. The dressing was changed twice a day for two days. On the third day the patient was discharged as completely improved. There has been no recurrence of symptoms since.

Case No. 9: P. J., S1c: This was one of our first cases on which crude penicillin gauze pads were used. Some six months before entry he had struck his right leg against a gun-casing causing a wide and deep abrasion which was treated with hot stupes, rest, sulfathiazole, etc. The ulceration failed to heal despite all treatment including hospitalization and repeated skin grafts. He had been in bed in this dispensary for about six weeks with little or no improvement. Penicillin gauze pads were applied several times daily and within one week the ulcer had reduced to one-half its original size. At the end of two weeks the patient was returned to duty completely healed.

Case No. 10: J. L., S1c: Entered the dispensary 9/1/44 with a large multiopening carbuncle on the mid-lumbar region. It was several inches in diameter, deep and painful. Radical incisions were made and all dead tissues removed under

intravenous anaesthesia. The following day penicillin gauze packs were packed into the wound and kept moist by frequent applications of crude penicillin solution. Within ten days the area had healed to the size of a dime and in three days more, the patient was returned to duty.

Case No. 11: One of our corpsmen reported to us one evening complaining of four boils on his right knee and leg which were very painful. One was opened and drained and penicillin gauze pads were applied to all four. The next day he stated that within an hour he had considerable relief from pain and slept comfortably. Within 48 hours all evidence of infection, swelling, redness and pain had disappeared and the patient's knee and leg were normal. Even the unopened boils subsided and pain left within a short time.

Case No. 12: R. E. W.: Infected cyst, teratoma, sacral region. Opened and drained. Improved with penicillin but became stationary. A gauze pad inoculated with *Torulospora utilis* variety *major,* a yeast-like organism prepared by Carpenter (4), was applied. In 24 hours the wound was clean and healed rapidly.

Case No. 13: Chronic indolent ulcer mid-portion of right shin caused by injury three months earlier. Laceration was infected and an indolent ulcer with induration around edge, size 1½ inches in diameter, was oozing pus and serum. Hot packs failed to help in one week. Penicillium gauze cleared up infection in twenty-four hours, and in ten days the wound was practically healed.

Case No. 14: Carbuncle, posterior neck with five small openings one-week duration. Very painful, exuding considerable pus and deeply indurated. Penicillium gauze packs first applied 10/10/44 and changed frequently. Pain was relieved in twenty-four hours; entire area softened up in forty-eight hours and all redness disappeared. On the fifth day the patient returned to work completely recovered.

Summary

1. Simple methods of production of crude penicillin solutions and inoculated gauze dressings are described, and their uses in topical therapy are discussed.

2. These penicillin preparations are cheap, easy to make, readily transportable, simple to use and markedly potent.

3. These preparations save the precious purified penicillin for the more serious generalized infections. They satisfy the need for penicillin in cases for which pure penicillin is not obtainable.

4. Clinical observations in fourteen typical cases are reported.

Acknowledgment:

I wish to take this opportunity to express my appreciation to the members of the research laboratories of the Experiment Station, H.S.P.A., for the many hours of their time and their kindness in supplying materials and for the instruction of our corpsmen in their methods of penicillin production. Penicillin preparations produced by these methods have been used with such success in our dispensaries that I felt they should be made available to all interested naval medical activities. The writer is especially grateful to the Experiment Station, H.S.P.A., for the publication of this manuscript.

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Antibacterial Properties of Yeasts, *Fusarium* Sp., Onion and Garlic

By C. W. Carpenter

Associations and antagonisms among microorganisms, and certain antibacterial substances of microbial and vegetable origin, are discussed. A simple technique is described which revealed that not only the penicillin-sensitive Staphylococcus aureus *but also the penicillin-resistant pus-forming bacteria,* Pseudomonas aeruginosa (Bacillus pyocyaneus) *and* Escherichia coli (Bacillus coli), *were inhibited* in vitro *by yeasts and yeast-like organisms, and by the juices and fumes of onion and garlic. One salient feature of the antibacterial activity of yeasts is identified. Clinical cases are reported which tend to establish the antibacterial potency* in vivo *of the yeast-like organism* Torulospora utilis *var.* major. *In addition to the cases mentioned herein, Dr. Nils P. Larsen, in discussing Case No. 10 in this issue of* The Hawaiian Planters' Record, *comments favorably on the possibilities of topical yeast-therapy in stubborn infections. Albert R. Agmar, Lt. Comdr. MC-V(S), USNR, used Torulospora culture solution with favorable results in Case No. 12 of his series of selected cases, also published in this issue of* The Hawaiian Planters' Record. *The technique used for the preparation of dynamic yeast cultures and inoculated surgical dressings is discussed.* Staphylococcus aureus *was found to be sensitive to the culture solutions of a* Fusarium oxysporum *variety. Possible applications of the principle of microbial association and antagonism to those phases of the sugar industry wherein microorganisms are constructive or destructive factors are visualized.*

Foreword

Studies of microbial antagonism and the production of antibiotic substances by microorganisms while not closely related to sugar production may be rationalized as endeavors to contribute to the cultural and humane aspects of the industry. Two papers in this issue of *The Hawaiian Planters' Record* discuss, respectively, the growth of *Penicillium notatum* and the preparation of active solutions of penicillin, and clinical results obtained with topical applications of such solutions. The mycological products supplied by the Experiment Station or by technicians trained here have satisfied an urgent need for penicillin for external use in the plantation hospitals and by local physicians as well as medical officers of the armed forces during the period when pure penicillin was unavailable for civilian use and later when its use was stringently controlled.

The amazing success of penicillin suggests possible applications of the principle of microbial antagonism to those phases of our industry wherein microorganisms are constructive or destructive factors. The employment of penicillin and similar substances for the control of infections and diseases of plantation livestock may be anticipated. We may conjecture applications of the principle of antibiotics in the control of certain diseases of sugar cane and possibly in the control of insects in addition to the well-developed project of insects as parasites.

Paramount in interest, however, is the rôle of microbial association and antagonism in the decomposition of sugar cane residues in the soil in relation to the available nitrogen. The soil, we are informed, tends to be in a state of biological equilibrium but it may be thought to approach such a state only when newly incorporated sources of energy—*e.g.,* decomposable trash and roots—have been reduced to their lowest chemical forms. The decomposition of raw plant materials to humus is a complexity of such infinite detail that it challenges the imagination. Series of chemical changes succeed series as successive microbial systems rework the degraded material—sometimes in harmonious association but conceivably more often in the antagonistic disharmony of competition.

In estimating the amount of nitrogen to apply at a given time in the crop cycle, the need of understanding and if possible in some measure directing the activities of desirable microbial systems and suppressing unfavorable systems is only too evident. A better knowledge of microbial associations and antagonisms may contribute eventually to the development of more refined techniques for estimating the amount of soil nitrogen which will become available in a definite time.

Association and Antagonism of Microorganisms

The phenomena of association and antagonism among microorganisms have been extensively investigated since Pasteur (24) in 1863 discussed the natural association of aerobic and anaerobic bacteria, and De Bary (5) in 1879 pointed out the antagonism incident to the competition of various bacteria coexisting in the same environment. Sooner or later some species survive at the expense of other less qualified types. The antagonistic activity of one organism toward another was termed antibiosis by Ward (38). Other descriptive designations have come into use for phases of antibiosis: antibacterial, inimical to bacteria; bacteriostatic, preventing bacterial multiplication; and bactericidal, deadly to bacteria.

Waksman (36) has presented a comprehensive review of microbial associations and antagonisms, with citations from 107 titles, to which the reader is referred for historical aspects of this interesting subject. In the present paper, only those investigations pertinent to the antibacterial propensities of yeasts and yeast-like organisms and the similar potency of certain vegetables can be cited, no reference to the antibacterial activity of *Fusarium* sp. having been found. The great majority of the papers cited have been available only as abstracts: the more significant papers were published in chemical rather than in bacteriological or mycological periodicals and regrettably were not found until the studies were nearly completed.

The observations discussed herein were made incidental to studies of the production of food yeast from molasses mashes and while also associated in a study of *Penicillium notatum*. The latter investigation, reported elsewhere, is currently significant since pure penicillin is still unavailable except under stringent regulations. Penicillin culture solutions and inoculated gauze surgical dressings, for topical use only, were prepared under the supervision of the medical adviser of the Association and supplied to plantation and other hospitals as well as to medical officers of the armed forces and to local physicians. To improve this service physicians were asked to identify pathogenic bacteria or to submit cultures for a test of their sensitivity to penicillin solution prior to initiating treatment. Some of these bacteria proved non-sensitive *in vitro*. Two of these organisms isolated from mixed infec-

tions with *Staphylococcus aureus* were identified by bacteriologists as the gram-negative types *Pseudomonas aeruginosa* (*Bacillus pyocyaneus*) and *Escherichia coli* (*Bacillus coli*). A search for some substance which might inhibit these organisms in a manner similar to the action of penicillin toward *S. aureus* was undertaken. The studies are discussed in three sections: first, preliminary observations which demonstrated the antibacterial activities of yeasts and *Torulospora* sp., *Fusarium* sp. and onion and garlic juices and vapors; a second section devoted to the antibacterial properties of the yeast-like organisms and the *Fusarium* sp.; and a third section wherein the antibiotic properties of onion and garlic are considered. The first and third sections are preceded by reviews of earlier investigations.

Antibacterial Properties of Yeasts and Yeast-like Organisms

The earliest mention found regarding antibacterial activity of yeast was by Sergent (28) in 1903 who reported a substance produced by yeast which was toxic to *S. aureus*. He may have cited earlier studies of this property of yeasts but his paper was not available for review. Burnett (4) in 1905 reported that yeast was being used as a disinfectant at the Nebraska Agricultural Experiment Station.*

Hayduck (14) observed a poisonous product developed by crushed rye, wheat and barley which was toxic to yeasts; he stated that he had prepared an active yeast poison from brewers' and compressed yeast. Fernbach (8) discussed substances produced by yeasts which were toxic to the same yeasts as well as to bacteria. He presented evidence that this substance was different from the cereal product of Hayduck. He also reported that the toxic mash was quickly overrun with molds, but that it preserved itself for a long time against invasion by other organisms which suggested to him that it might have antiseptic properties. To translate from Fernbach: "We have established experimentally that our mashes of yeast have very strong antibacterial properties." In his tests the neutralized fluid was filtered through a porcelian bougie and the filtrate tested in cultures of *B. coli* and *S. pyogenes aureus*. Fernbach stated that the substance was volatile when distilled *in vacuo* at 40° C., the residue being inactive; the active principle was destroyed at 100° C. He also stated that he obtained an active toxin by distillation of fresh yeast *in vacuo* in a small amount of water, an experiment which was held to exclude the idea that the antibacterial substance did not preexist in the yeast but had formed during desiccation.

Hayduck (15, 16) questioned some of the results of Fernbach's investigations, in particular the volatility of the toxic substance, whereupon Fernbach and Vulquin (10) continued the studies. They refer to an earlier paper (9), saying in effect: "We have already shown that the antibacterial action of soaked yeast . . . is due to a volatile substance having the reaction of the amines. Although the nature of this amine escaped us, we are able to assert that the toxic substance isolated by us as crystals from hydrochloric acid resembles the complex amines because we have observed no antibacterial power in the simple amines, neither those containing a

* The procedure was substantially as follows: One cake of either compressed or dried yeast was moistened with water and allowed to stand for 16 to 24 hours, and then one to one-and-a-half pints of lukewarm water were added. This mixture was used as a cleansing fluid to destroy disease-producing bacteria and was found especially promising in cases of contagious abortion of cows.

simple alcohol radical, nor those substituted one, two, or three times.... We have arrived at the conclusion that the toxic substances of the mashes of yeast and cereals are different in nature." The authors presented evidence that the distillate from wheat mash was active in suppressing yeast growth only in the presence of sugar while the distillate from yeast mash was not thus dependent.

Kraemer (20, p. 49) mentioned a commercial yeast product for the treatment of putrid wounds, ulcers, etc., marketed under the name Xerase. He gave the formula as follows: dried yeast 100 parts; dextrose 20 parts; white clay or aluminum silicate 125 parts; nutrient salts 3 parts. Feriz (7) reported that dried yeast was dusted upon inflamed tonsils with favorable results. Skchiwan (30) according to Guilliermond (13, p. 122) reports that the yeasts have practically no chemiatric action on the leucocytes of animals. The same author (31) found that phagocytosis of the yeast cells occurred but the latter were demonstrated to be alive by inoculation into culture media. Rettger, Reddish and McAlpine (25) reported that bakers' yeast when ingested by man and the white rat underwent a rapid and extensive destruction in the alimentary tract with less than one per cent survival of the cells. The injection of pure suspensions of bread yeast into white mice, guinea pigs and rabbits by the subcutaneous, intravenous and intraperitoneal routes failed to show

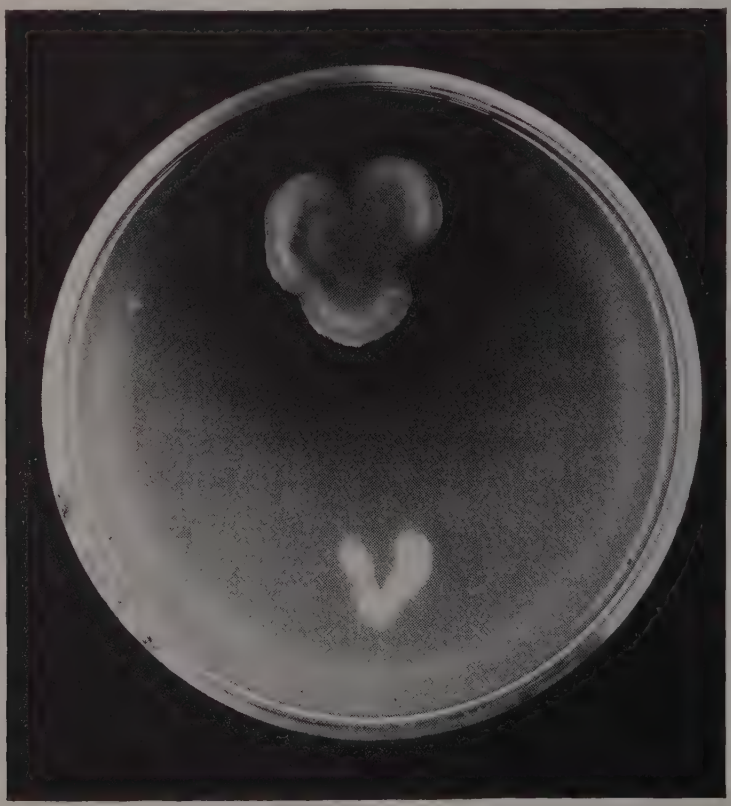


Fig. 1. Above: Lysis (dissolving action) of *Staphylococcus aureus* by *Pseudomonas aeruginosa* (*B. pyocyaneus*). Below: No lysis by *Escherichia coli*. (Plantings of the two bacteria were made on a 24-hour growth of *S. aureus* and the plates incubated.)

evidence of injury aside from the formation of a small, firm nodule in two of the twenty-one animals employed. No suppuration or necrosis ensued.

Schiller (26) distinguished between natural antagonism and the antagonism induced when two organisms are cultured together. When wine or beer yeast are grown with bacteria in the presence of sugar and no protein they become antagonistic—the bacteria are digested by a proteolytic substance. The lytic substance (27) in induced antagonism is never produced in pure culture while in natural antagonism (*e.g.,* pyocyanase by *B. pyocyaneus*) it always is (Fig. 1). Three experiments are mentioned in which the lytic substance produced by yeast, growing in association with tubercle bacilli, was injected together with the pathogen into guinea pigs; favorable indications—*i.e.,* a slower progress of the induced disease—are reported in comparison with the usual sequela.

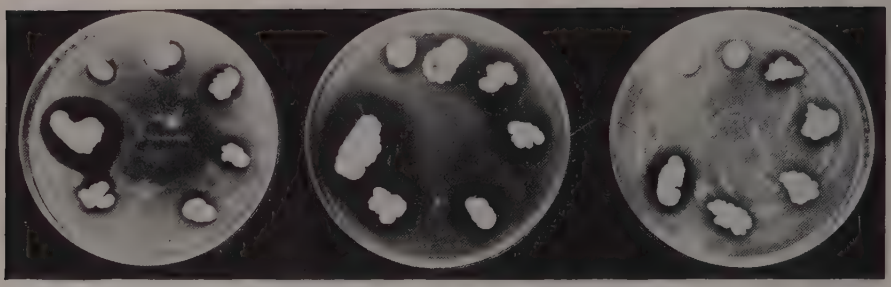
Henrici (17, p. 231), alone on the contrary, states that yeasts have no demonstrable antibacterial action. He does not mention the investigations of Fernbach and Hayduck or other investigators or any experimental evidence in explanation of this statement. No refutation of the work reported by Fernbach (10) and his associate was found in the literature.

Bachmann and Ogait (2) reported that the antibacterial action of yeast was due to the large amounts of acetaldehyde produced. Tikka and Itkonen (33) stated that a thermolabile material toxic for *B. aerogenes* was formed by yeast in bread dough. Takahashi (32) reported the extraction of a substance from brewers' and bakers' yeasts, by autolysis and autoclaving, which was capable of inactivating the virus of tobacco mosaic. This substance was found stable to heat and, therefore, concluded not to be an enzyme.

The observations and experimental evidence cited above are sufficiently convincing to justify a much more exhaustive study of the antibacterial property of yeasts and yeast-like organisms than the writer has thus far undertaken. Further information on the nature of the substance which inhibits bacterial growth and the mode of operation is desirable. The very limited clinical tests which have been conducted with the cooperation of local physicians indicate beneficial effects when yeast preparations are applied topically for eliminating such secondary pus formers as *Ps. aeruginosa* and *E. coli* after topical applications of penicillin have controlled the primary infection caused by *S. aureus*. It seemed important to learn if the substance is produced in pure culture of the yeast (natural antagonism) or only in the presence of another organism (acquired antagonism). It was likewise important to know if the substance could be concentrated, isolated and purified, and if so, whether it was toxic to body cells.

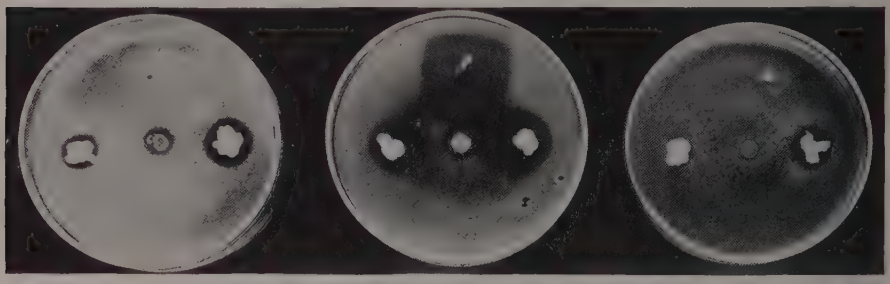
Preliminary Search for an Antibacterial Substance Effective Against Gram-Negative Rod-Forms of Bacteria

A simple technique was employed to detect possible antibacterial power of various organisms and materials of microbial and plant origin. Poured plates of the three pathogens, *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Escherichia coli,* were prepared as a daily routine and on these cultures the organisms or materials to be tested were superimposed as transplants (Figs. 2, 3), or in glass or plastic



Ps. aeruginosa *S. aureus* *E. coli*

Fig. 2. Comparative inhibition of *Ps. aeruginosa, S. aureus,* and *E. coli* by seven yeasts and yeast-like organisms.



Ps. aeruginosa *S. aureus* *E. coli*

Fig. 3. Comparative inhibition of *Ps. aeruginosa, S. aureus,* and *E. coli* by a food yeast, a beer yeast and *T. utilis* var. *major,* in a horizontal line across the plates, and *Penicillium notatum* above.

Oxford cylinders* (Fig. 4) to simulate the chance contamination of Fleming's (11) plates by *Penicillium notatum.* These plates were incubated at 37° C.

The organisms and materials tested included many isolates of unidentified species of Penicillium and Aspergillus, one or more isolates of *Rhizopus nigricans, Neurospora sitophila, Helminthosporium sacchari, Fusarium oxysporum* var. undetermined (parasitic on *Opuntia megacantha*), *Trichoderma* sp., *Saccharomyces cerevesiae, S. ellipsoideus,* and other true yeasts, the yeast-like organisms *Torulospora utilis* and its varieties *thermophila* and *major;* the enzymes available—diastase, emulsin, pepsin and trypsin; also the juices of papaya fruit, tomato, beet, celery, cabbage, potato, onion, and garlic, as well as the vapors of the two latter vegetables in inverted petri-dish cultures. *P. notatum* was placed on many of the plates for comparison.

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* The assay of the antibacterial potency of liquids was conducted by the modification of the cup-plate method devised by Heatley (1) in which cylinders of standard dimensions, *i.e.,* 9.6 mm. long, 5.1 mm. inside diameter and 7.2 mm. outside diameter, are set on the surface of plate cultures of the test bacteria, and filled with the liquid to be assayed. After incubation at 37° C. each cylinder which had contained bacteriostatic liquid was surrounded by a clear zone where the bacteria had not developed. The diameter of this inhibition zone is a function of the bacteriostatic efficiency of the liquid.

Fig. 4. Inhibition of *Ps. aeruginosa* (*B. pyocyaneus*) and *S. aureus* by culture solution of *Torulospora utilis* var. *major*.' At left: Undiluted solution in Oxford cylinders. At right: Solution diluted with an equal amount of sterile water.

In these preliminary studies, it was found that *S. aureus* was inhibited not only by *P. notatum* but by one strain of *Penicillium* isolated from a ripe banana, the *Fusarium oxysporum* var. above mentioned, the yeasts, the *Torulospora* sp. and varieties, and the juices and vapors of onion and garlic. The gram-negative *Ps. aeruginosa* and *E. coli* were inhibited only by the *Torulospora* sp. and varieties, the

yeasts, and the onion and garlic material. *T. utilis* var. *major* was the most active inhibitor among the yeasts and yeast-like organisms, the zones of inhibition of *S. aureus* approaching in diameter those formed by *Penicillium notatum* on the same plates; with *Ps. aeruginosa* and *E. coli* the zones were only slightly smaller. It was observed that young cultures of *T. utilis* var. *major* were more active than older cultures and that the medium on which the test was conducted exerted an important influence on the inhibitory effect. Garlic juice and vapor were much the most active of the vegetable materials tested and with respect to the three pathogens much more potent against *E. coli* than against the other two. Isolations attempted to recover *E. coli* and *S. aureus* from the inhibition zones failed, indicating that for them the garlic materials were bactericidal.

Control transplants were made in the tests using commercial yeast extract, thiamine chloride, dry but viable yeast, invertase and ethyl alcohol (2 to 20 per cent in Oxford cylinders). No inhibition of the bacteria by any of these materials was observed.

Since the tests were conducted at 37° C., a temperature at which the yeasts grow very slowly, and the tests were usually completed in 16 to 24 hours, it was tentatively presumed that the inhibitory action of these organisms might be due either to a labile substance, preformed on a favorable medium, which did not persist in old cultures, or to a substance produced even during the limited growth of the cells at the unfavorably high temperature.

Experiments designed to probe the nature of the observed antibacterial property of the yeasts and yeast-like organisms were conducted, the two varieties of *T. utilis, thermophila* and *major,* being used for this purpose in the majority of the tests. Information was sought on the following salient points: (1) Locale of the active substance, whether in the cells or closely associated therewith, or in the culture solution. (2) Mode of action of the inhibitory factor; relation of sugar and fermentation to the production of the active substance. (3) Reaction of the substance to heat; if labile to heat, an enzyme or volatility of the substance would be logical indications. (4) Relation of the hydrogen-ion concentration of culture media to the production and performance of the substance. A series of representative experiments selected to illustrate the observations made in the investigation of these four salient features is reported in detail.

Comparative Potency of Yeast Cells and Culture Solution

Cultures of *T. utilis* var. *thermophila* and var. *major* were prepared in liquid media, the cells precipitated by centrifuging and tests of the relative antibacterial activities of the latter and of the supernatant culture fluid were made. Sucrose and dextrose were used respectively as energy sources in a synthetic medium prepared according to the following formula:

MEDIUM 1

Distilled water	1,000 ml.
$(NH_4)_2SO_4$	2 gm.
K_2HPO_4	1 gm.
Yeast extract (Difco)	5 gm.

To one-liter portions of the medium 20 gm. of white sugar (Medium A), 20 gm. raw sugar (Medium B) and 20 gm. dextrose (Medium C), were added respectively.

Two 500-ml. portions of each medium were disposed in one liter prescription bottles which were then plugged with cotton and also equipped with pipettes plugged with cotton filters for aeration of the media during incubation. After autoclaving, one bottle of each medium was inoculated with sterile water suspensions of cells of *T. utilis* var. *thermophila* and var. *major,* respectively. The units were incubated in a water bath at 30° C. for 40 hours during which time the media were constantly aerated.

A sample of each culture was then removed with a sterile pipette and centrifuged ten minutes at 1800 r.p.m. Three plates of *S. aureus* were prepared and four Oxford cylinders set on each one. The cylinders were then filled respectively with precipitated yeast and supernatant liquid of each of the organisms from each of the three media. The inhibition zones observed after incubation of the plates at 37° C. are recorded in Table I.

TABLE I

DIAMETERS OF INHIBITION ZONES OF *S. AUREUS* PRODUCED BY YEAST CELLS AND SUPERNATANT SOLUTION

		Media		
		A	B	C
T. utilis var. *thermophila*	Liquid	25 mm.	27 mm.	40 mm.
	Cells	20 mm.	25 mm.	40 mm.
T. utilis var. *major*	Liquid	11 mm.	11 mm.	16 mm.
	Cells	17 mm.	30 mm.	22 mm.

Another sample of yeast and liquid was withdrawn from the bottom of one of the bottles of *T. utilis* var. *major* for further study. Tests were made as before with five samples obtained during repeated centrifuging as follows: (1) Supernatant liquid after first centrifuging; the liquid was poured off and the still moist yeast centrifuged again; the small amount of liquid (2) and the moist yeast (3) were tested; discarded the supernatant liquid; twenty ml. of sterile water were added to the precipitated yeast cells and mixed therewith; centrifuged once more; the supernatant wash water (4) and the washed cells (5) were tested. The five listed materials were tested in Oxford cylinders on plates of *Ps. aeruginosa* and *S. aureus.* The diameters of inhibition zones recorded after 24 hours incubation at 37° C. are shown in Table II.

TABLE II

INHIBITION OF *PS. AERUGINOSA* AND *S. AUREUS* BY YEAST CULTURE SOLUTION, WASH WATER AND CELLS

	First Centrifuging	Second Centrifuging		Third Centrifuging	
	Supernatant liquid	Supernatant liquid	Cells	Wash water	Cells
Ps. aeruginosa	21 mm.	21 mm.	20 mm.	15 mm.	21 mm.
S. aureus	20 mm.	15 mm.	20 mm.	20 mm.	20 mm.

Apparently, the supernatant culture solution and the water used to wash the precipitated yeast cells were as potent as the cells in antibacterial effect. The experiment was not sufficiently refined to eliminate the possibility that in all cases the yeast may have been present in viable condition in the Oxford cups and grew sufficiently therein, even at 37° C., to produce an antibacterial substance.

Mode of Operation of the Inhibitory Factor of Yeasts and Yeast-like Organisms

Relation of sugar to the inhibitory factor:

Poured plates of *Ps. aeruginosa, S. aureus* and *E. coli* were prepared with a sugar-free medium made according to the following formula:

MEDIUM 2

Distilled water		1,000. ml.
Bacto nutrient agar (Difco)		23. gm.
Containing: Bacto beef extract	3. gm.	
Bacto peptone	5. "	
Bacto agar	15. "	
Neopeptone (Difco)		5. gm.

Test plantings of *T. utilis* var. *thermophila, T. utilis* var. *major,* a beer yeast and a food yeast (No. 0–87) were made on the plates and the latter were incubated at 37° C. No indication of inhibitory activity was observed.

To a reserved portion of this medium, 2 per cent dextrose was added and the medium tubed and resterilized. The inhibitory activity was in evidence when the bacteria were tested on this modified medium. This experiment indicated that sugar was required in the medium on which the test bacteria were grown for the manifestation of the antibacterial effect. However, it was suspected that the resterilization of the medium after the dextrose was added might have increased the hydrogen-ion concentration and that the two media were not identical in this respect.

To examine further the apparent dependence of *T. utilis* var. *major, T. utilis* var. *thermophila* and food yeast No. 0–87, on sugar for the exhibition of antibacterial activity, a medium, to portions of which various amounts of dextrose were added, was prepared as follows:

MEDIUM 3

Distilled water	1,000. ml.
$(NH_4)_2SO_4$	2. gm.
K_2HPO_4	1. "
Peptone (Difco)	5. "
Yeast extract (Difco)	5. "
Bacto agar (Difco)	15. "

A portion of this medium was reserved as a sugar-free control and to other portions one, three and five per cent dextrose were added, respectively, prior to sterilization. Plates of *S. aureus* were prepared on the four media. Test plantings of *P. notatum,* the *Torulospora utilis* varieties and the yeast No. 0–87 were made on the plates which were then incubated for 16 hours at 37° C. The diameters of the incubation zones observed are recorded in Table III.

TABLE III

INHIBITION OF *S. AUREUS* BY *T. UTILIS* VARIETIES AND YEAST NO. 0–87 ON MEDIA WITH AND WITHOUT DEXTROSE

		Dextrose		
	No dextrose	1%	3%	5%
P. notatum	40 mm.	—	38 mm.	38 mm.
T. utilis var. *thermophila*	—	—	22 mm.	25 mm.
T. utilis var. *major*	—	—	18 mm.	22 mm.
Yeast No. 0–87	—	slight	15 mm.	22 mm.

This experiment provided further indication that sugar was necessary to the manifestation of inhibition of *S. aureus* by the yeast and yeast-like organisms. Although the media were adjusted to pH 5.5 prior to sterilization, it is reasonable to assume that those with the higher percentages of dextrose may have become slightly more acid than the control or the medium containing one per cent dextrose.

Influence of Dextrose and the pH Values of the Test Medium on Inhibition

Two series of five media at different pH values were prepared according to the formula of Medium No. 2—one series containing dextrose and one series without. The pH of each medium determined electrometrically after sterilization is shown in Table IV. Poured plates of each medium were prepared with *Ps. aeruginosa, S. aureus* and *E. coli,* respectively, and on each plate two Oxford cylinders were placed. One cylinder on each plate was then filled with a liquid culture of *T. utilis* var. *major* and the other with succinic acid—borax buffer at pH 3.5. Cells of *T. utilis* var. *thermophila* and *major* were also transplanted on to each plate. The plates were then incubated 16 hours at 37° C. The diameters of the inhibition zones observed are shown in Table IV wherein media Nos. 1–5 were sugar-free and Nos. 6–10 contained 2 per cent dextrose.

TABLE IV

INHIBITION OF *PS. AERUGINOSA* BY *TORULOSPORA UTILIS* VAR. *THERMOPHILA* AND *MAJOR* AND BY SUCCINIC ACID—BORAX BUFFER pH 3.5

	Medium	pH	*T. utilis* var. *major* liquid	Succinic acid-borax buffer pH 3.5	*T. utilis* var. *major* cells	*T. utilis* var. *therm.* cells
Sugar-free	1	4.54	—	—	—	—
	2	5.05	22 mm.	20 mm.	slight	slight
	3	5.05	25 mm.	22 mm.	11 mm.	11 mm.
	4	5.40	20 mm.	11 mm.	—	—
	5	5.57	11 mm.	—	—	—
2% dextrose	6	4.45	25 mm.	15 mm.	15 mm.	15 mm.
	7	5.07	12 mm.	10 mm.	8 mm.	8 mm.
	8	5.15	10 mm.	—	trace	trace
	9	5.40	12 mm.	15 mm.	trace	trace
	10	5.52	20 mm.	10 mm.	slight	—

STAPHYLOCOCCUS AUREUS

	Medium	pH	*T. utilis* var. *major* liquid	Succinic acid-borax buffer pH 3.5	*T. utilis* var. *major* cells	*T. utilis* var. *therm.* cells
Sugar-free	1	4.54	15 mm.	15 mm.	—	—
	2	5.05	15 mm.	14 mm.	—	—
	3	5.05	15 mm.	12 mm.	—	—
	4	5.40	12 mm.	13 mm.	—	—
	5	5.57	20 mm.	9 mm.	—	—
2% dextrose	6	4.45	19 mm.	16 mm.	11 mm.	10 mm.
	7	5.07	15 mm.	10 mm.	8 mm.	7 mm.
	8	5.15	18 mm.	15 mm.	7 mm.	8 mm.
	9	5.40	15 mm.	10 mm.	trace	trace
	10	5.52	16 mm.	11 mm.	trace	trace

ESCHERICHIA COLI

	Medium	pH	*T. utilis* var. *major* liquid	Succinic acid-borax buffer pH 3.5	*T. utilis* var. *major* cells	*T. utilis* var. *therm.* cells
Sugar-free	1	4.54	30 mm.	28 mm.	—	—
	2	5.05	15 mm.	17 mm.	12 mm.	14 mm.
	3	5.05	19 mm.	19 mm.	12 mm.	10 mm.
	4	5.40	11 mm.	12 mm. (partial)	trace	—
	5	5.57	15 mm.	18 mm.	—	—
2% dextrose	6	4.45	20 mm.	—	trace	trace
	7	5.07	20 mm.	18 mm.	trace	trace
	8	5.15	15 mm.	12 mm.	trace	trace
	9	5.40	16 mm.	12 mm.	trace	?
	10	5.52	15 mm.	12 mm.	—	—

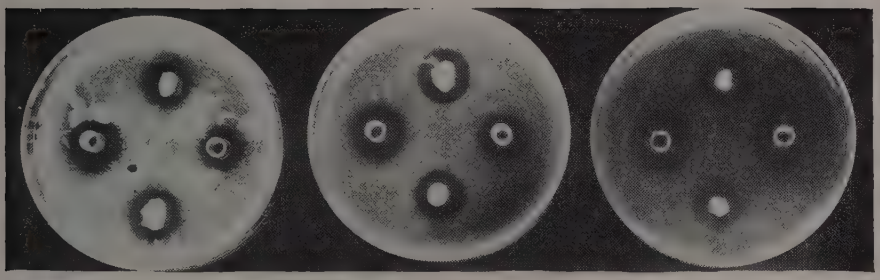
Ps. aeruginosa grew on all the media. In the presence of dextrose there was superior inhibition in Medium No. 6 (pH 4.45) and in Medium No. 10 (pH 5.52) when compared respectively with Medium No. 1 (pH 4.54) and Medium No. 5 (pH 5.57), without dextrose. In the absence of dextrose, there was superior inhibition in Medium No. 2 (pH 5.05), in Medium No. 3 (pH 5.05) and in Medium No. 4 (pH 5.40) in comparison respectively with Medium No. 7 (pH 5.07), No. 8 (pH 5.15), and No. 9 (pH 5.40), with dextrose.

S. aureus grew more vigorously on all of the media than either *Ps. aeruginosa* or *E. coli,* the differences being more marked in the media with pH values 5.05–4.54. Inhibition zones produced by the liquid culture of *T. utilis* var. *major* and by the buffer solution were comparable at each pH value regardless of the presence or absence of dextrose in the medium. However, the cells of the two yeasts caused no inhibition in the absence of dextrose; they caused a moderate inhibition in the presence of dextrose at pH values 4.45, 5.07 and 5.15. This observation indicated that the cells carried little of the inhibitory substance, but developed it by metabolic activity in the presence of dextrose.

E. coli also grew on all the media. The inhibition of this organism by the liquid culture of *T. utilis* var. *major* and by the buffer solution was much the same as with *S. aureus* with respect to the influence of dextrose on the manifestation of the activity. However, the inhibition caused by the transplanted cells was much more marked in the absence of dextrose than in its presence—the reverse of the condition noted with *S. aureus.*

Inhibition of *Ps. aeruginosa, S. aureus* and *E. coli* by Sterile Succinic Acid-Borax Buffer Solutions

Two succinic-acid-borax buffer solutions were prepared at pH values, which it was presumed might produce approximately the same degree of inhibition of the test bacteria as *T. utilis* var. *major* if acid production by the yeast was an active component of the antibacterial effect. Prior to sterilization, these buffer solutions were pH 2.98 and 4.02; after autoclaving, they were respectively pH 3.42 and 4.42. Plates of the bacteria were prepared, Oxford cylinders placed thereon and filled with the buffer solutions. The diameters of the inhibition zones (Fig. 5) observed after incubation at 37° C. are shown in Table V.



Ps. aeruginosa *S. aureus* *E. coli*

Fig. 5. Comparative inhibition of three pathogenic bacteria: At bottom, by *T. utilis* var. *major* plantings; at top, by var. *thermophila;* at left, in cylinder, by succinic acid-borax buffer pH 3.42; and at right, by pH 4.42.

TABLE V

INHIBITION OF *PS. AERUGINOSA, S. AUREUS* AND *E. COLI* BY SUCCINIC ACID-BORAX BUFFERS

Buffer	*Ps. aeruginosa*	*S. aureus*	*E. coli*
pH 3.42	21 mm.	27 mm.	12 mm.
pH 4.42	20 mm.	25 mm.	0

Succinic acid solutions were prepared at the following concentrations: 1.0, 0.5, 0.25 and 0.125 per cent. The 1.0 per cent solution had a pH value of 3.0 (La Motte Yellow indicator). These solutions when tested against *S. aureus* in Oxford cylinders on a medium at pH 5.5 formed inhibition zones as follows: 1.0 per cent 17–22 mm.; 0.5 per cent 16–20 mm.; 0.25 per cent 10 mm.; 0.125 per cent 0 mm.

The 1.0 per cent succinic acid solution and an active yeast culture solution failed to inhibit *S. aureus* after they had been neutralized with sodium hydroxide solution (Bromthymol Blue indicator). Titratable acid of 1.0 ml. of the unheated yeast solution (pH 3.0) was equivalent to 1.2 c.c. of N/20 NaOH (Bromthymol Blue indicator).

Influence of Amount of Sugar in Culture Medium in Which *T. utilis* var. *major* Had Developed on Inhibition of *E. coli*

A test was conducted to determine if the concentration of sugar in the medium in which *T. utilis* var. *major* had grown influenced the antibacterial potency of the cells toward *E. coli.* Fulmer and Nelson's sugar-free inorganic medium, to portions of which sucrose was added in the percentages 1, 2, 5 and 10, was prepared as follows:

Distilled water	1,000 ml.
$(NH_4)Cl$	1.8 gm.
K_2HPO_4	1.0 "
Bacto agar (Difco)	15.0 "
pH 6.6	

These four media, after tubing, sterilizing and slanting, were inoculated with *T. utilis* var. *major* and incubated two days at room temperature (25–30° C.). Portions of the yeast growth were planted on plates of *E. coli* in a synthetic agar

medium at pH 5.5. The incubation zones formed after 24 hours at 37° C. were as follows: Cells from 1 per cent sucrose medium, 15 mm.; 2 per cent sucrose medium, 17 mm.; 5 per cent sucrose medium, 17 mm.; and from the 10 per cent medium, 15 mm. Regardless of the amount of sucrose in the nutrient medium on which the yeast had grown, the inhibition of *E. coli* was practically the same.

Antibacterial Effect of Viable vs. Pasteurized Cells of *T. utilis* var. *major*

A sample of heavy yeast suspension was removed from the bottom of a liquid culture of *T. utilis* var. *major* and equal portions of it placed in each of two sterile tubes. One portion was heated in a water bath at 75° C. for five minutes. This portion was then immediately cooled and plated to test its viability; it was subsequently found to be non-viable.

After the heated sample had been cooled, Oxford cylinders were installed in a plate of *S. aureus* and filled respectively with the heated and the unheated slurry. After incubation, it was observed that the unheated yeast had produced inhibition zones 15 mm. in diameter while the heated slurry had been entirely inactivated. This test indicated that either the antibacterial substance of the yeast was volatile, heat labile, or that living cells were essential to the manifestation of inhibition.

Culture solution of *T. utilis* var. *major,* which manifested strong antibacterial potency, was found to maintain this potency after heating in boiling water for five minutes. Viable cells of the organism undoubtedly were not responsible for this inhibition, but the latter was due to a substance already present in the solution. When cells are transplanted onto plates of the pathogens, sufficient growth and production of the substance probably occurs at 37° C. to cause inhibition; in the case of culture solutions, substances preformed during incubation cause a similar effect.

Relation of the pH Value of the Medium on Which the Test Bacteria are Grown to Inhibition

It had become evident in the experiments discussed above and in other tests that the hydrogen-ion concentration of the medium on which *Ps. aeruginosa, S. aureus* and *E. coli* were grown was significant to the consistent manifestation *in vitro* of inhibition when yeast-like organisms were being studied. A survey was made of pH values which might be unfavorable for these species. Medium No. 3, to which two per cent dextrose and one per cent sucrose were added, was prepared and seven portions of it adjusted colorimetrically, prior to sterilization, to the pH values shown in Table VI. After sterilization, slanted cultures of *Ps. aeruginosa, S. aureus,* and *E. coli* were made in duplicate on each of the seven media. Table VI shows the range of growth of the pathogens observed after incubation at 37° C. The pH values of media Nos. 4 to 6 determined electrometrically with uninoculated media are shown at the bottom of the respective columns of the table. The behavior of the three organisms differs in this experiment from that observed in the preceding experiments where all grew at pH 4.45, likewise in the presence of sugar.

TABLE VI

pH VALUES WHICH LIMIT THE GROWTH OF *PS. AERUGINOSA, S. AUREUS* AND *E. COLI*

Medium Orig. pH	1 3.5	2 3.8	3 4.3	4 4.7	5 5.3	6 5.7	7 6.5
Ps. aeruginosa	—	—	—	—	+	+	+
S. aureus	—	—	—	trace	+	+	+
E. coli	—	—	—	—	+	+	+
pH (Electrometric)				4.99	5.48	6.00	

Sierakowski and Milejkowska (29) list the upper and lower pH values which are lethal for many pathogenic bacteria, sixteen of which are given in Chemical Abstracts 19: 90–91. These authors state that pH 3.8 is fatal for *E. coli* and pH 3.9 for *S. aureus.*

Three liquid cultures of *T. utilis* var. *major,* originally at pH values 5.5–6.0 were found to have pH values 2.98, 2.72 and 3.15 respectively when tested after two days incubation at 30° C.

The Non-volatile Nature of the Antibacterial Substance of *Torulospora utilis* var. *major* and Commercial Yeast

The method described by Fernbach (8) and by Fernbach and Vulquin (9) by which they claim to have demonstrated that the antibacterial substance of yeasts was volatile was tried in a preliminary rather than an exhaustive manner. Heavy suspensions of commercial yeast (*Saccharomyces cerevesiae*) and *Torulospora utilis* var. *major* were distilled at reduced pressure at 40–50° C. and the distillates tested respectively for antibacterial activity. None was detected. Fernbach's (8) and Fernbach's and Vulquin's (9) procedure wherein the cells of yeast were dried rapidly at 37° C. and 2 gm. taken up in 200 cc. of 1–1,000 hydrochloric acid, incubated and "neutralized exactly to alizarine sulfaconjugaée" was also attempted. Aliquots of the hydrochloric acid mixture were adjusted colorimetrically to several pH values approaching neutrality, *i.e.,* pH values 5.4–7.0, since "alizarine sulfaconjugaée" was not available as an indicator. The only experiment conducted failed to give any indication that this method would prove successful with the organisms used. These observations should not be regarded as conclusive especially in view of Fernbach and Vulquin's positive statements on the volatility of the substance and its isolation as crystals from hydrochloric acid.

Torulospora utilis var. *major* and *Penicillium notatum* are Mutually Non-antagonistic

Plates of *T. utilis* var. *major* and *P. notatum* were prepared with a medium containing dextrose. Test plantings of *P. notatum* were made on the plates of *T. utilis* var. *major* and similarly the latter organism was implanted on the plates of *P. notatum.* After several days incubation at room temperature, no evidence of antagonism was observed on the part of either organism toward the other. In topical applications apparently, preparations of these organisms might be used simultaneously. However, the penicillin might be partially inactivated by the acids produced by the yeast.

Antagonism of *T. utilis* var. *major* Toward Bacteria Is Natural Rather Than Acquired

According to Schiller's (*26, 27*) concept, microbial antagonism may be differentiated into natural antagonism and acquired antagonism; in the former, the antibacterial principle is produced in pure culture, and in the latter, antibacterial activity only develops when organisms grow in association. He states that when yeasts are grown with bacteria in media containing sugar but no protein the yeasts become antagonistic and the bacteria are digested by a proteolytic substance.

The antibacterial activity of yeasts in several of the experiments recorded above was observed in media containing peptone as well as sugar. Since a portion of the antibacterial action of yeasts *in vitro* may, in view of the experiments discussed, be attributed to the production of acid in the course of sugar fermentation, and this acid is formed in pure culture, we should refer to their antibacterial action as natural antagonism.

An experiment was conducted with *Ps. aeruginosa, S. aureus* (local strain and Oxford strain) and *E. coli,* each grown alone and each also grown in association with *T. utilis* var. *major.* Two synthetic non-protein media, *i.e.,* Cohn's solution and Nawiasky's basal salt solution (modified) were each prepared in part with two per cent dextrose and in part without dextrose. The formulae of the four media were as follows:

MEDIUM A

Cohn's solution:

Distilled water	1,000. ml.
K_2HPO_4	5. gm.
$MgSO_4$	5. "
Ammonium tartrate	10. "
KCl	.5 "

MEDIUM B

Medium A	500. ml.
Dextrose	10. gm.

MEDIUM C

Nawiasky's basal salt solution (modified):

Distilled water	1,000. ml.
$(NH_4)Cl$	5. gm.
K_2HPO_4	2. "
$MgSO_4$	.5 "

MEDIUM D

Medium C	500. ml.
Dextrose	10. gm.

The four media were adjusted to pH 6.8.

After tubing and autoclaving, two tubes of each medium were inoculated respectively with one of the four bacteria mentioned. One tube of each medium which had been inoculated with one of the test bacteria was then also inoculated with *T. utilis* var. *major* so that a culture of each bacterium and a culture of the same bacterium growing in association with the yeast on each of the four media were available for study. After incubating the cultures for two weeks at room temperature, streak transfers were made from each tube onto dextrose nutrient agar (pH 6.8) to note the viability of the bacteria. The effect of *T. utilis* var. *major* upon

the viability of each of the four bacteria when grown in association therewith is summarized for each organism as follows:

Ps. aeruginosa: This bacterium suppressed the growth of *T. utilis* var. *major* in Cohn's solution both in the sugar-free and in the two per cent dextrose media. In Nawiasky's solution, without dextrose, the same condition prevailed. Pyocyanase production was the logical cause of the antibiotic effect. In the latter medium, with dextrose, the bacteria had been so inactivated by the yeast that no growth developed from the streak inoculation. This might be interpreted as a vindication of Schiller's concept. The yeast grew luxuriantly in the streak inoculation.

Staphylococcus aureus (Oxford strain): In Cohn's solution, *T. utilis* var. *major* suppressed the coccus in the absence of dextrose as well as in its presence. In Nawiasky's solutions, this coccus was not entirely inactivated except in the solution containing dextrose, an observation which again supported Schiller's concept.

S. aureus (local strain): In Cohn's solution, in the absence of dextrose, the yeast apparently had little effect on the coccus; with dextrose, the coccus was suppressed but not entirely prevented from making some growth in the streak culture. In Nawiasky's solutions, the coccus was eliminated only in the medium containing dextrose.

E. coli. In both Cohn's and Nawiasky's solutions, this organism was incapacitated by the yeast for growth in the streak culture only in the solutions which contained dextrose.

In the two media containing dextrose wherein the four test bacteria and *T. utilis* var. *major* were cultured in association, the latter organism consistently prevented or greatly suppressed the growth of the bacteria. That this effect was partly due to the production of acid by the yeast was indicated by the pH values in the tubes concerned which, by colorimetric determinations, averaged about pH 3.0. Schiller's (*26, 27*) concept of acquired antagonism postulates that yeasts dissolve the bacteria growing in association with them, the lysis being effected by a proteolytic substance —the necessary conditions being the presence of sugar and the absence of protein. These are also the conditions which may be construed to favor the accumulation of acids in the medium as a result of fermentation. Protein is not essential for the growth of yeast but is favorable, if not essential, for the growth of many bacteria.

Inhibition of *Staphylococcus aureus* by *Fusarium oxysporum* var. Undetermined

Inhibition of *S. aureus* was observed when a variety of *Fusarium oxysporum*, parasitic on the common prickly pear (*Opuntia megacantha*), was planted on plates of the bacteria. The fungus was grown on Czapek-Dox medium and the culture solution tested in comparison with *Penicillium notatum*. Inhibition zones up to 35 mm. in diameter were observed when the fungus was grown in a small amount of culture solution but, in comparison with *P. notatum* in larger amounts of solution, it was not nearly as potent.

It is mentioned to record antibacterial power in another genus of fungi apparently not hitherto reported, since other species of this large and diverse genus may be found more potent, either on the medium mentioned or on a medium more suitable for their growth.

Resumé:

Yeasts are frequently mentioned in the lore of ancient *materia medica.* Only in the last few decades has their specific value in diets in deficiency diseases, furunculosis, abscesses, boils, etc., been correctly interpreted and their beneficial action ascribed to their content of vitamins essential in nutrition. During the current interest in avitaminosis, other possible beneficial properties such as antibacterial potency may not have been sufficiently scrutinized.

In view of the considerable number of affirmative reports concerning the antibacterial properties of yeasts, supported by experimental evidence and the apparent lack of refutation of the positive statements of several investigators, it is reasonable to presume that some of the yeasts and yeast-like organisms may be capable of producing antibacterial substances to a greater extent than might be concluded from the experiments described herein.

The *Torulospora* sp. studied in the experiments showed a greater inhibitory potency *in vitro* than several true yeasts. The experiments with two varieties of this species indicated that a substantial part of the inhibition of *Ps. aeruginosa, S. aureus* and *E. coli* could be attributed to the formation of an acid in a medium containing sugar which reduced the pH value of the medium to pH 5 or lower. In one experiment none of these organisms grew appreciably on an agar medium at pH 4.99. It is suggested that yeast-culture solution diluted with an equal amount of sterile five per cent dextrose solution applied topically would, on account of its dynamic nature, be superior to acid solutions or acid buffers, which are essentially static. The clinical use of dynamic yeast culture in five cases of purulent infection, including one infected cyst and four cases of secondary infection with *Ps. aeruginosa* and *E. coli,* after *S. aureus* had been eliminated by topical applications of culture solutions of *Penicillium notatum,* has indicated that the material is not only non-toxic but is remarkably efficient in topical applications. *Torulospora utilis* var. *major,* which was used in these tests by local and service physicians in cases not responding to conventional treatments, grows sufficiently at the unfavorably high temperature of 37° C. to produce the antibacterial effect.

The activity of the varieties of Torulospora when grown in association with the above-mentioned pathogens in media containing sugar but no protein tended to support Schiller's (26, 27) concept that under such conditions certain yeasts become antagonistic to bacteria. The conclusion is equally tenable that precisely these conditions favor the yeasts more than the bacteria and are ideal for fermentation with the release of various substances, one of which is an acid. A culture solution of *T. utilis* var. *major,* which had been tested and found to inhibit the pathogens, yielded a gelatinous precipitate, with a solution of ferric chloride, indicating the presence of succinic acid.

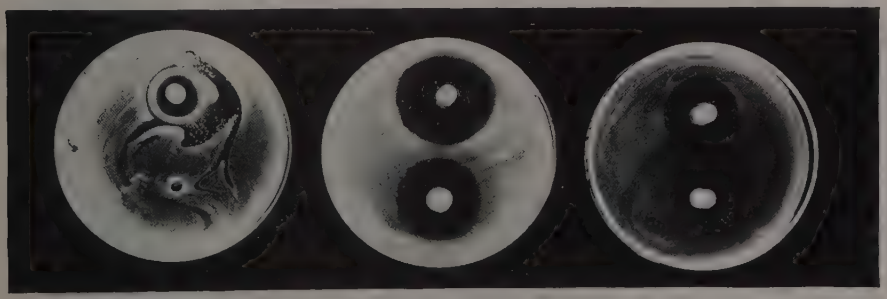
In the Oxford-cylinder method of assay (see footnote p. 46) the diameter of the inhibition zone is a function of the bacteriostatic efficiency of the solution being tested. Obviously the size of the inhibition zone is fundamentally dependent upon the rate and extent of diffusion of the inhibitory substance into the agar. It would be hazardous to conclude by analogy that inhibition zones of equal size, produced under the same conditions by a complex yeast-culture solution and a simple acid or buffer solution were due to the same inhibitory factor, or that the acid solutions and the yeast culture would have comparable effects on pus-forming bacteria when applied topically.

Antibacterial Properties of Onion and Garlic

The earliest experimental work on the antibacterial properties of garlic found in the literature was that of Dombray and Vlaikovitch (6) in 1924. This paper was not available and earlier investigations may have been cited. The authors prepared an alcoholic extract of garlic which checked the growth of several bacteria; they concluded that the alcohol in the extract could not account for the observed effect. Minchin (23) used garlic in studies of tuberculosis and reported favorable indications. Lehmann (21) found that the juice of garlic inhibited the growth of *B. proteus,* prevented the decay of meat, and was lethal for *Paramecium caudatum,* and some worms. Walton, Herbold and Lindegren (37) also observed the bactericidal potency of garlic and found that this property was destroyed by heat in boiling and autoclaving. Kitagawa and Amano (18) studied the effect of garlic on *Escherichia coli, in vitro,* and *Ebertella typhosa, in vivo* (mouse) and noted indications of strong antiseptic powers. Kitagawa and Hirano (19) in a study with *E. coli* determined the phenol coefficient of garlic alkylpolysulfid to be 15 in comparison with mercuric chloride 115.

Vollrath, Walton and Lindegren (35) reported that acrolein (allyl aldehyde) or some related unsaturated aldehyde is possibly the active bactericide in garlic. Lovell (22) stated that onion vapors were bactericidal but less active than those of garlic and more rapidly dissipated, while Foter and Golick (12) observed that the inhibitory effect of horse-radish was greater than that of garlic and onion, more rapidly exhausted than that of garlic but more persistent than that of onion. Bocher (3) commented on the volatile nature of the active principle of garlic and observed that the vapors were effective at a distance of 20 cm. above the freshly crushed vegetable. Several of the mentioned contributors to the subject reported that these vegetables were much more effective at a temperature of 37° C. than at lower temperatures.

The research of Dr. B. Tokin (34), University of Tomsk, U.S.S.R., was briefly reviewed in *Science News Letter* under the title: "Onion Paste Helps Wounds." Two five-minute exposures of wounds to the vapors of freshly crushed onions were reported to have checked serious infections and hastened healing. The antibac-



Ps. aeruginosa *S. aureus* *E. coli*

Fig. 6. Comparative inhibition of *Ps. aeruginosa, S. aureus,* and *E. coli:* upper zones, by diluted garlic juice (1 to 1); and below, active culture solution of *Penicillium notatum.* The latter inhibited only *S. aureus.*

terial substances in onions, garlic and other vegetables are called phytonicides by Dr. Tokin.

As mentioned previously the inhibitory power of onion and garlic was observed in the course of routine studies of various microbial and plant materials in a planned search to find some substance which would be effective against *E. coli* and *Ps. aeruginosa,* organisms unaffected by penicillin (Fig. 6). The studies of the bactericidal efficiency of the juices of these vegetables confirm the earlier investigations mentioned above.

Inhibition of *Ps. aeruginosa, S. aureus* and *E. coli* by Onion and Garlic Juices

Fresh onion juice in Oxford cylinders superimposed on plate cultures of *S. aureus* inhibited the growth of the latter moderately, the diameter of the inhibition zones averaging 15 mm. after 24 hours incubation at 37° C. Portions of the same juice stored for 24 hours in the refrigerator were found to have lost all potency. In another test wherein the activity of onion and garlic juices toward *S. aureus* was compared, the former again produced inhibition zones 15 to 20 mm. in diameter while fresh garlic juice diluted with more than an equal amount of sterile distilled water formed zones more than 45 mm. in diameter (Fig. 7). Such garlic juice stored in the refrigerator three days and diluted with sterile water 1–1 and 1–10 produced inhibition zones on plates of *Ps. aeruginosa, S. aureus* and *E. coli,* the diameters of which are shown in Table VII. At a dilution of 1–75 no inhibition was observed.

TABLE VII

INHIBITION OF *PS. AERUGINOSA, S. AUREUS* AND *E. COLI* BY REFRIGERATED AND DILUTED GARLIC JUICE

	Dilution	
	1–1	1–10
Ps. aeruginosa	25 mm.	trace
S. aureus	40 mm.	25 mm.
E. coli	28 mm.	21 mm.

The inhibition zones observed in the experiments with fresh onion and garlic juices confirm Lovell's (22) report that garlic juice is more potent and retains its power longer than onion juice.

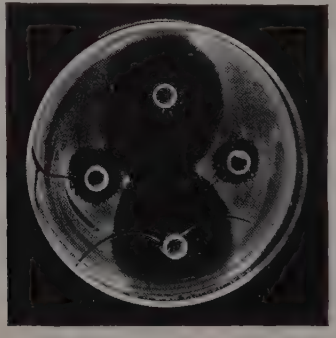


Fig. 7. Comparative inhibition of *S. aureus:* upper and lower fusing zones of inhibition by garlic juice; and zones at right and left by onion juice.

Surgical Dressings for Vapor Treatment of Infections

Extracts from freshly crushed garlic were prepared and used to moisten surgical dressings which were tested *in vitro* against *Ps. aeruginosa, S. aureus* and *E. coli.* It was thought that if these dressings should prove to have inhibitory power they would be convenient for use. Two 20-gm. portions of freshly crushed garlic were extracted for 10 minutes with absolute alcohol and carbon tetrachloride, respectively. The filtrates were used to moisten sterile gauze dressings in petri dishes which were then refrigerated. After two days the dressings were taken from the refrigerator, and with the covers of the dishes removed, the solvent remaining in the gauze was evaporated under a fan. Portions of the dressings approximately 1 cm. square were cut with sterile scissors; one series was left dry while another was moistened with sterile normal saline solution. The portions of the dressings were then planted on poured plates of the above mentioned bacteria. The diameters of the inhibition zones observed after 16 hours incubation at 37° C. are shown in Table VIII.

TABLE VIII

INHIBITION OF *PS. AERUGINOSA, S. AUREUS* AND *E. COLI* BY GAUZE IMPREGNATED WITH GARLIC EXTRACTS

	Alcoholic extract		Carbon-tetrachloride extract	
	Dry	Saline	Dry	Saline
Ps. aeruginosa	15 mm.	15 mm.	12 mm.	—
S. aureus	35 mm.	35 mm.	20 mm.	20 mm.
E. coli	40 mm.	38 mm.	33 mm.	35 mm.

Duplicate dressings were tested after three months refrigeration at which time those prepared with the carbon tetrachloride extract had lost all inhibitory power while those made with the alcoholic extract formed inhibition zones with diameters as follows: *Ps. aeruginosa* 20 mm.; *S. aureus* 30 mm.; and *E. coli* 30 mm. (Fig. 8).

Freshly crushed garlic was extracted with chloroform and the filtrate agitated in a separatory funnel. After standing for a few hours a milky suspension had settled out below a clear supernatant liquid. Sterile gauze dressings were moistened with the two fractions, respectively, and dried under a fan. Portions of the two dressings were cut and tested as above described. The clear supernatant liquid exhibited no antibacterial power. The diameters of the inhibitory zones formed by the milky fraction were recorded as follows: *Ps. aeruginosa* 25 mm.; *S. aureus* 35 mm.; and *E. coli* 40 mm.

These dressings were prepared and tested with the idea that such preparations might be useful for fume treatment if direct application proved irritating, as might be suspected. If they should prove non-irritating *per se,* the normal saline would tend to allay any irritation caused by a dry dressing.

Garlic Vapor Lethal for Certain Insects

The lethal effect of garlic vapors on several insects was observed by placing them in covered dishes with freshly crushed material. Contact of the insects with the garlic was prevented by means of plastic screen. Honey bees, blowflies, greenbottle flies and soldier flies (*Hermetia illucens*) succumbed in an hours time while control insects confined in the same amount of air space lived 10 hours or more. These observations supplement the report of Lehmann (21) on the lethal power of garlic vapors on *Paramecium* sp. and some worms.

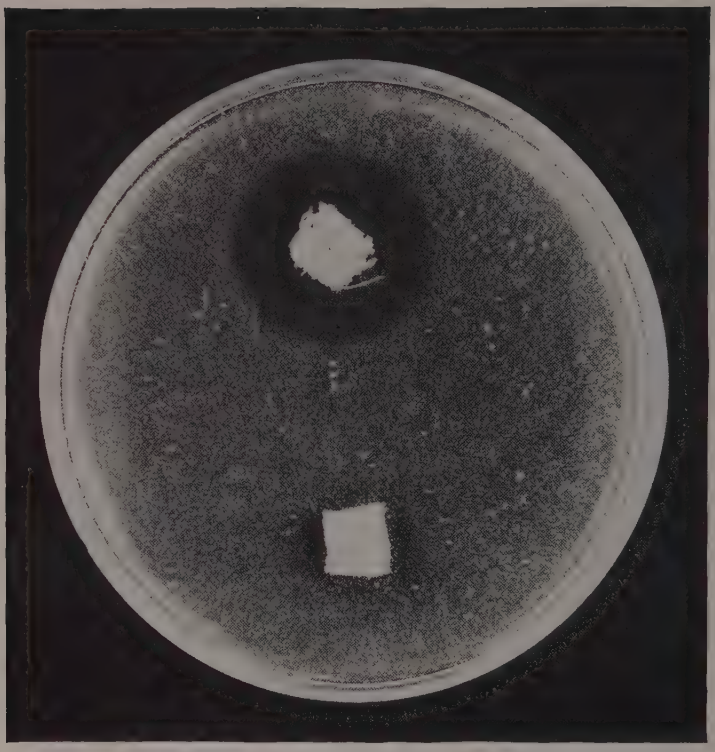


Fig. 8. Inhibition of *S. aureus:* at top, by portion of a gauze dressing wet with alcoholic extract of garlic and refrigerated three months; and below, no inhibition from portion of dressing wet with carbon tetrachloride extract and refrigerated the same length of time.

The Volatile Nature of the Antibacterial Substance of Garlic

The volatility of the antibacterial substance of garlic was demonstrated as follows: Poured plate cultures of *Ps. aeruginosa, S. aureus* and *E. coli* were prepared in duplicate, one series being a control; the other series was inverted but not sealed, over "Spray" anaerobic culture dishes; in each of the latter a few grams of freshly crushed garlic had been placed, about 4 cm. below the cultures. The plates were incubated at 37° C. The control cultures developed normally in 24 hours while the cultures over the garlic remained free of all growth even after 48 hours incubation. When the latter were removed from the influence of the garlic emanations, only *Ps. aeruginosa* grew after further incubation.

In a similar experiment, duplicate sets of bacterial plates were prepared and the petri dishes inverted; in the centers of the covers of one set about 15 mm. below the agar surface, small portions of crushed garlic were placed. After incubation for 24 hours at 37° C. inhibition zones directly above the pieces of garlic measured as follows: *Ps. aeruginosa* 30 mm.; *S. aureus* 40 mm.; and *E. coli* 42 mm. in diameter (*cf.* Fig. 9).

Fig. 9. Inhibition of *S. aureus* and *E. coli* in plates inverted over two small portions of garlic. Control plates below.

Resumé:

The antibacterial potency of onion and garlic observed by several investigators was confirmed for *Ps. aeruginosa, S. aureus* and *E. coli, in vitro*. Garlic fumes were lethal for honey bees, blowflies, green-bottle flies and soldier flies (*Hermetia illucens*). The practical use of onion and garlic in controlling infections has apparently received little attention. The report of the clinical use of such materials in Russia by Dr. Tokin suggests that more attention may be devoted to this subject in the future.

Summary

1. The studies of antibiotic substances suggest the possible application of the principle of microbial association and antagonism to those phases of our industry wherein microorganisms are constructive or destructive factors.

2. A better knowledge of microbial association and antagonism may contribute to the eventual development of more refined techniques for estimating the amount of soil nitrogen which will become available in a definite time.

3. A simple technique for detecting antibiotic substances is described.

4. *Torulopsis utilis* and its varieties *thermophila* and *major* and, to a lesser degree, certain true yeasts inhibit not only *Staphylococcus aureus* but also the gram-negative bacteria *Pseudomonas aeruginosa* (*B. pyocyaneus*) and *Escherichia coli* (*B. coli*) *in vitro*.

5. In those cases where yeast and *Torulospora* cells were planted on the plates, the inhibition of the three mentioned pathogens was found to be dependent upon the presence of sugar in the medium upon which the bacteria were grown. Culture solutions of the yeasts or the yeast-like organisms often inhibited the growth of these bacteria in the absence of sugar in the test medium due to the accumulation of antibiotic substances in the solutions.

6. The rapid evolution of an acid or acids, one of which was identified as succinic acid by test with ferric chlorid solution, accounts in part for the observed inhibitory activity toward the three pathogens. When yeast cells are planted on plates of the test bacteria, in media containing sugar, sufficient growth of the yeast occurs even at 37° C. to produce inhibition zones.

7. Inhibition of the three pathogens comparable with that produced by the yeasts and yeast-like organisms was induced *in vitro* with succinic acid—borax buffer solution at pH 3.5 and with solutions of various acids. Culture solutions of *T. utilis* var. *major* originally with pH values 5.5 to 6.5 were found after two days incubation to have values from 2.98 to 3.15. None of the pathogens grew on one medium at values below pH 5.

8. Preliminary clinical tests indicate that yeast-culture solution diluted with an equal amount of sterile, five per cent dextrose solution, when applied topically for purulent infections, constitutes a dynamic means of inhibiting the growth of *Ps. aeruginosa, S. aureus,* and *E. coli* and probably many other organisms which are intolerant of an acid medium. Acid solutions and the acid buffers similarly used are essentially static.

9. Such a dynamic culture of *T. utilis* var. *major* has been used clinically by local and service physicians with success in cases of infected cyst and other purulent infections, etc., the solution being applied topically. It has been of value in several cases to clear secondary infections after the primary infection with *S. aureus* had been eliminated with topical applications of culture solutions of *Penicillium notatum*.

10. The activity of *Torulospora utilis* varieties, when grown in association with *Ps. aeruginosa, S. aureus,* and *E. coli* respectively, in media containing sugar but no protein tended to support Schiller's (26, 27) concept that, under such conditions, certain yeasts become antagonistic and the bacteria are digested by a proteolytic substance. The conclusion is equally tenable that precisely these conditions favor fermentation of the sugar by the yeasts with the release of various substances and the development of an acid reaction unfavorable to the bacteria.

11. Failure of a preliminary experiment designed to demonstrate that the yeasts studied produce a volatile antibacterial substance should not be regarded as refutation of Fernbach and Vulquin's positive statement that such a product was produced

by the yeasts they studied and that this substance was isolated as crystals from hydrochloric acid. Some yeasts may produce antibacterial substances to a greater extent than might be concluded from the experiments discussed.

12. *Fusarium oxysporum* (var. undetermined), parasitic on the common prickly pear (*Opuntia megacantha*), inhibited the growth of *S. aureus, in vitro,* in a manner similar to *Penicillium notatum.* This is apparently the first observation of antibacterial potency of a species of *Fusarium* against a human pathogen.

13. The antibiotic properties of onion and garlic observed have been reported previously by several investigators. Garlic vapors were found to be lethal for *S. aureus* and *E. coli* and bacteriostatic for *Ps. aeruginosa.* The vapors were found lethal for such insects as honey bees, green-bottle flies, blowflies and soldier flies (*Hermetia illucens*). The researches of Russian scientists followed by the clinical use of onion vapors for wound infections indicates that further investigation of the antibacterial properties of these and other plants may be expected.

14. The occurrence of antibacterial substances in onions and garlic and the production of substances having antibacterial potency by fungi of several genera as well as by various species of bacteria, and the observed antibacterial activity of yeasts and yeast-like organisms *in vitro,* seem to extend infinitely the possible sources from which such substances may be obtained. The progress already made in the field of microbial antagonism encourages studies of the chemical means by which various organisms employ offense as a weapon of defense, successfully compete with one another or dominate their environment, and survive. Superimposed planting of materials on poured agar plates of pathogenic bacteria and the Oxford-cylinder method offer techniques for discovering additional sources of antibiotic substances.

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Sugar Prices

96° CENTRIFUGALS FOR THE PERIOD
SEPTEMBER 16, 1944, TO DECEMBER 15, 1944

Date	Per pound	Per ton
Sept. 16–Dec. 15, 1944.....	3.75¢	$75.00

Sugar prices on page 307 of the Fourth Quarter 1944 *Record* should read:

Date	Per pound	Per ton
June 16–Sept. 4, 1944.....	3.74¢	$74.80
Sept. 5–Sept. 15, 1944.....	3.75¢	75.00

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